

# **HIV INFECTION IN SUB - POPULATIONS OF CD4+ LYMPHOCYTES DEFINED BY CD45.**

**Matthew Reginald Helbert**

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fulfilment of the requirements for the degree of Ph.D

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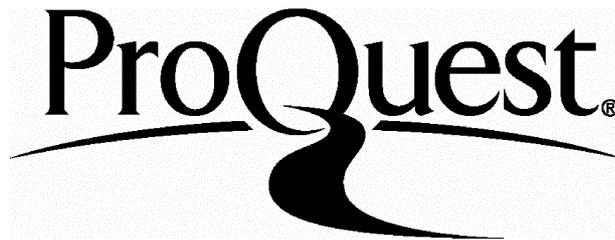
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## Abstract; HIV INFECTION IN SUB - POPULATIONS OF CD4+ LYMPHOCYTES DEFINED BY CD45.

Cardinal features of HIV infection are clinical loss of immunological memory and failure of CD4+lymphocytes to respond to recall antigens *in vitro*. CD4+ lymphocytes expressing the RO isoform of CD45 mediate immunological memory and are lost early in infection and infected more readily by HIV; the aim of this project was to investigate these predilections for CD45RO+ cells.

CD4+ lymphocytes expressing CD45RO and CD45RA (antigen naive cells) express similar quantities of CD4, the receptor for HIV, and bind the viral envelope glycoprotein, gp120, equally well. Gp120 binding induces weak activation signals in both populations, reflected by influxes of calcium, somewhat greater in CD45RO+ cells. Gp120 also induces down - regulation of CD4, particularly in CD45RA+ cells; CD45RO+ cells may be protected from this effect by tyrosine kinase activity. CD45RO+ cells form syncytia with chronically infected T cell lines more efficiently than their CD45RA+ counterparts, possibly a consequence of increased integrin expression and affinity.

*In vitro*, cell free HIV infection also precedes more effectively in CD45RO+ cells, possibly because reverse transcription proceeds more readily in the activated cells contained in this population. When CD45RA+ and RO+ cells are activated *in vitro* following infection, there are similar amounts of viral DNA in both populations, implying a degree of basal activation in un - stimulated CD45RO+ cells promotes cell free infection.

TNF has been cited as a means of breaking viral latency in lymphocytes, particularly those expressing CD45RO. However, resting CD45RA+ and RO+ cells express minimal quantities of TNF receptor and are unable to transduce TNF signals. The "TNF autocrine loop" may not be important in up - regulating HIV transcription in resting cells.

HIV may also indirectly affect the population kinetics of CD4+ lymphocytes, by affecting antigen presenting cell function; the differences shown here between CD45RA+ and CD45RO+ populations are not major, but together may contribute towards the understanding of the preferential infection of CD45RO+ cells.

**Science is an articulated structure of hypotheses and has no place for, or need of, absolute truth<sup>5</sup>.**

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## List Of Abbreviations.

AIDS	Acquired immunodeficiency syndrome
ARAM	Antigen recognition activation motifs
ATP	Adenosine tri - phosphate
BSA	Bovine serum albumen
cAMP	Cyclic adenosine mono - phosphate
CD	Cluster of differentiation
CDR	Complementarity determining region
CLA	Cutaneous lymphoid antigen
DAG	Di - acyl - glycerol
DC	Dendritic cell
DNA	De - oxy - ribo - nucleic - acid
dNTP	De - oxy - nucleotide - tri - phosphate
EMSA	Electromotility shift assay
FACS	Fluorescence activated cell sorter
FITC	Fluorescein - iso - thiocyanate
FIV	Feline immunodeficiency virus
gag	Genome associated antigen
GCSF	Granulocyte colony stimulating factor
GMCSF	Granulocyte - macrophage colony stimulating factor
gp120	HIV envelope 120 kD glycoprotein
HEV	High endothelial venule
HIV	Human immunodeficiency virus
HTLV1	Human T lymphotropic virus 1
ICAM	Inter - cellular adhesion molecule
IFN	Interferon
IL	Interleukin
IP	Inositol polyphosphate
$K_d$	Dissociation constant
LCK	Lymphocyte tyrosine kinase
LFA	Leukocyte functional antigen
LPS	Lipopolysaccharide
LTR	Long terminal repeat
mAb	Monoclonal antibody
MCB	Monochlorobimane
MHC	Major histocompatibility complex
MnSOD	Manganous superoxide dismutase
MOI	Multiplicity of infection
MRC-ADP	Medical Research Council AIDS Directed Programme
NF $\kappa$ B	Nuclear factor $\kappa$ B (originally identified in B cells expressing $\kappa$ light chains)
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction

PHA	Phytohaemagglutinin
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol myristic acid
RNA	Ribo - nucleic - acid
ROI	Reactive oxygen intermediaries.
RT	Reverse transcription
SF	Syncytium formation
SIV	Simian immunodeficiency virus
TcR	T cell receptor
TGF	Transforming growth factor
TH	T helper cell
TK	Tyrosine kinase
TNF	Tumour necrosis factor
VLA	Very late antigen
ZAP70	Zeta associated peptide

# Chapter 1; Immunological Memory And T Cell Activation.

## 1.1.0. Cells In The Immune System; Overview.

The primary roles of the immune response are the surveillance, recognition and elimination of infectious agents. Innate immunity is not enhanced by repeated exposure to antigen. The adaptive response, by contrast, mounts an improved response on re-exposure, a consequence of specificity and immunological memory.

Key cells in the innate immune response include neutrophils, monocyte/macrophages and dendritic cells (DCs). Neutrophils and monocytes have the ability to phagocytose organisms whilst monocytes and, particularly, DCs present antigen to and support the more highly evolved adaptive immune response, which is mediated by T and B lymphocytes. The role of B cells is to recognise antigen, using surface expressed immunoglobulin molecules, and to differentiate subsequently into plasma cells to produce large quantities of immunoglobulin. B cells have the unique ability to undergo somatic mutation, the result of which can be production of higher affinity immunoglobulin. The maintenance of the B cell - or humoral - response is dependent on help from T<sub>H</sub>2 cytokine secreting T cells, whilst B cell memory is maintained by antigen retained on the surface of follicular DCs.

T cells are lymphocytes which have passed through a phase of thymic differentiation and are a more heterogeneous population than B cells; cells expressing CD8 respond to antigen presented in the context of Major Histocompatibility Complex Class I (MHC Class I) molecules. These cells mediate cytotoxicity, restricted by the MHC Class I molecule and T cell receptor specificity. CD8 cells can also inhibit viral replication without cytotoxicity<sup>1</sup> and may have a role in suppressing aspects of the immune response.

T cells expressing the CD4 molecule are discussed below. Both CD4 and CD8 cells exist as either virgin or memory cells. Virgin T cells have completed thymic maturation but not yet encountered their corresponding antigen in the periphery. Virgin T cells with specific T cell receptors exist at low frequency and require special conditions to become activated *in vivo*. Following an encounter with antigen in the appropriate co-stimulatory *milieu*, clones of activated T cells emerge from the progeny of virgin T cells; the majority of these appear as effectors of the immune response, whilst some persist as memory T cells. These are clonally expanded and have less critical activation requirements and so secondary exposure to antigen results in an enhanced, or an amnestic response.

### 1.1.1. Dendritic Cells.

DCs present antigen to all T lymphocytes. They are derived from CD34+ bone marrow precursor cells and their production is favoured by GM-CSF, IL1 or G-CSF in combination with TNF $\alpha$  and  $\beta$ , conditions which favour DC rather than monocyte colony formation<sup>2, 3</sup>. After leaving the marrow, DCs take on a series of site specific morphological changes. Migration from marrow to tissue is concomitant with the acquisition of dendritic morphology; in the skin DCs are known as Langerhans cells, migrating out of the skin to lymph node they assume a veiled cell morphology and in lymph node as interdigitating cells in the T dependent areas<sup>4</sup>. It is not clear whether interdigitating cells can return to peripheral tissues.

The large size and presence of dendrites may enhance their ability to present antigen since, unlike B cells, DCs can cluster with several T cells simultaneously. This allows cross talk between T cells responding to different antigens and from different T cell subsets<sup>5</sup>.

Blood DCs express low levels of co-stimulatory molecules such as MHC Class II<sup>6</sup>, ICAM-1, B7.1, B7.2<sup>421</sup> and LFA-3, but up-regulate these after *in vitro* culture<sup>7</sup>, involvement in allogeneic responses<sup>8</sup> and exposure to  $\gamma$ IFN and GM-CSF<sup>9</sup>. *In vivo*, the epidermal location of Langerhans cells facilitates their role as sentinel cells. Tissue DCs express more co-stimulatory molecules than the blood precursors<sup>7, 9</sup>. On exposure to antigen/hapten or cytokines secreted by dermal fibroblasts<sup>422</sup> DCs leave the epidermis and migrate to lymph node. In the mouse this is a systemic event; local antigen application results in a generalised efflux of DCs from the skin (S. Knight, personal communication).

The high level of co-stimulatory molecules on DCs, particularly B7<sup>7</sup> and MHC Class II molecules allows them to present antigen to both virgin and memory T cells<sup>10-13, 423</sup>.

Because DCs, unlike monocytes and B cells, present antigen to naive T cells, they play a key role in the shaping of an immune response. The presence of B7 on DCs favours the production of cytokines characteristic of T<sub>H</sub>1 cells, including  $\gamma$ IFN, in T cell progeny activated in this way<sup>14</sup>;  $\gamma$ IFN in turn up-regulates B7 expression<sup>9</sup>. In the presence of IL10, secreted by keratinocytes and T<sub>H</sub>2 cells, this positive T<sub>H</sub>1 feedback loop is disrupted<sup>15</sup>; T<sub>H</sub>1 cells become anergic<sup>16</sup>. In the case of monocytes, at least, IL10 down-regulates B7<sup>17</sup>.

Defects in DCs whether a reduction in number, migration or co-stimulatory molecule expression can thus result in both quantitative and qualitative changes in T cells<sup>18, 19</sup>.

## 1.1.2. Monocytes.

Like DCs, monocytes are derived from CD34+ bone marrow precursors. They are able to mature into macrophages which may survive in tissues for many years in more or less specialised states, for example glial cells in the brain. Unlike DCs, monocytes are able to present antigen only to memory lymphocytes<sup>10</sup>, but have additional effector roles in secreting cytokines and in phagocytosis.

## 1.1.3. T Lymphocytes.

T lymphocytes are defined as cells derived originally from the bone marrow which proceed through a maturational process in the thymus before emerging in the periphery. These cells all express CD2 (see 1.2.4) and CD3 (see 1.2.2). CD4+ cells support the adaptive immune response and are thus known as helper cells. CD4+ T cells can be functionally divided into clones which provide help for predominantly either the cellular (T<sub>H</sub>1) or for humoral (T<sub>H</sub>2) immune responses.

## 1.1.4. T<sub>H</sub>1 and T<sub>H</sub>2 CD4+ T Lymphocytes.

Subsets of CD4+ T cells can be distinguished, most readily in mice, by their repertoires of cytokine secretion. T<sub>H</sub>0 cells develop into either T<sub>H</sub>1 clones, secreting  $\gamma$ IFN and TNF (and increased IL2 in mice) or T<sub>H</sub>2 clones secreting predominantly IL4, IL5, IL6 and IL10. T<sub>H</sub>1 clones provide help for monocytes whilst T<sub>H</sub>2 clones help B cells (reviewed in<sup>20</sup>).

The distinct effects of these subsets are illustrated in the finding that *Leishmania major* results in a self limiting infection in inbred mouse strains which produce T<sub>H</sub>1 clones and fatal infection when T<sub>H</sub>2 clones predominate.  $\gamma$ IFN promotes the growth of T<sub>H</sub>1 clones and when anti -  $\gamma$ IFN is administered at the same time as *Leishmania* infection in resistant mice, T<sub>H</sub>2 clones are produced with a fatal outcome<sup>21</sup>. When T cells are cloned (using allogeneic stimulation) from mice infected with either *Brucella* or *Nippostrongylus*, there is enhancement of T<sub>H</sub>1 or T<sub>H</sub>2 clones, respectively<sup>22</sup>. These murine data imply that genetic restriction and different micro - organisms regulate, through early cytokine secretion, the type of T cell clone produced. When polyclonal activators are used in mice not stimulated *in vivo*, it has been more difficult to show these patterns<sup>23</sup>, although a third population of T<sub>H</sub>0 cells emerges, with a very restricted cytokine repertoire.

In humans, atopy provides an opportunity to study the impact of antigen presentation on cytokine secretion. Atopic patients produce T<sub>H</sub>2 clones in response to *Dermatophagoides* and T<sub>H</sub>1 responses to antigens not involved in atopy<sup>24</sup>. Other antigens, for example mycobacterial peptides, consistently yield T<sub>H</sub>1 clones in all donors<sup>25</sup>. These data have been used to suggest that the murine data applies to humans<sup>26</sup> although other workers have found that T cells cloned by polyclonal activation from

normal humans rarely have such distinct cytokine secretion patterns<sup>27</sup>. In fresh T cells stimulated for less than 24 hours cytokines are secreted predominantly by CD45RO+ cells but do not give a clear T<sub>H</sub>1/T<sub>H</sub>2 segregation with 1 in 4 IL4 secreting cells also secreting  $\gamma$ IFN<sup>28</sup>.

The genetic factors determining T<sub>H</sub>1/T<sub>H</sub>2 predisposition has not been identified, but it appears that the cytokine secretion by and presence of co - stimulatory molecules on cells of the innate immune system at the time of stimulation determines whether response is predominantly T<sub>H</sub>1 or T<sub>H</sub>2<sup>20</sup>. Infection with *Staphylococcus* or *Toxoplasma gondii* results in IL12 secretion by monocytes, which stimulates secretion of  $\gamma$ IFN by NK and possibly  $\gamma\delta$  cells.  $\gamma$ IFN and IL12 stimulate preferential growth of T<sub>H</sub>1 clones. IL4, IL1, TGF $\beta$  and IL10 all favour T<sub>H</sub>2 clones and are possibly secreted by mast cells early in the response<sup>29</sup>.

Whilst T<sub>H</sub>1 derived  $\gamma$ IFN stimulates B7 expression on macrophages<sup>30</sup>, T<sub>H</sub>2 derived IL10 reduces monocyte B7 expression<sup>17</sup>. IL10 also appears to reduce the ability of DCs to promote functional T<sub>H</sub>1 clones<sup>15, 16</sup>.

All activated T cells express surface bound TNF $\alpha$  and the CD40 ligand (CD40L); the counter - receptors for these molecules are homologous with one another, but restricted in distribution so that TNF receptors are expressed on macrophages and CD40 on B cells. These co - stimulatory molecules offer another means by which presenting cells can promote either T<sub>H</sub>1 or T<sub>H</sub>2 clones<sup>29</sup>. Hence, innate immunity can shape the response of the adaptive system (discussed in<sup>21</sup>).

## 1.2. T Cell Surface molecules.

### 1.2.1. T Cell Receptor.

The T cell receptor (TcR), a member of the immunoglobulin super family, consists of a dimer of  $\alpha$  and  $\beta$  chains (or, less frequently,  $\gamma\delta$  and  $\alpha\alpha$ ). These two chains are responsible for recognising antigenic peptide on either specific MHC Class I or II and hence impose antigenic and MHC specificity. The TcR is homologous to an immunoglobulin Fab fragment with each chain containing a constant (C) and variable (V) domain, constrained by intramolecular disulphide bridges.

The diversity of the TcR is achieved by recombinant events taking place in the thymus. Germ line V, joining (J) and diversity (D) genes (in TcR $\beta$  only) recombine and in doing so incorporate frame shift mutations which promote diversity. Although there is no peripheral somatic mutation in T cells, TcR affinity can mature at the population level through competition of different clones for antigen, rather than at the molecular level seen in B cells.

The intracytoplasmic parts of the TcR molecule are short and do not contain signalling domains. Hence signalling through the TcR relies on a second molecule, CD3.

## 1.2.2. CD3

CD3 and TcR are linked non-covalently, but require the presence of one another for surface expression. CD3 consists of monomeric  $\gamma$ ,  $\delta$ , and  $\epsilon$  chains associated with either  $\eta\zeta$  or  $\zeta\zeta$  dimers;  $\eta$  and  $\zeta$  are obtained by differential splicing of a gene distinct from the other CD3 chains. The  $\zeta$  chain is also used by CD2 for signalling<sup>31</sup>. The cytoplasmic tails of  $\gamma$  and  $\delta$  contain single antigen recognition activation motifs - ARAMs, whilst  $\zeta$  contains 3. These Y-X-L motifs are targets for phosphorylation by tyrosine kinase (TK) and subsequent binding by phosphotyrosine binding proteins<sup>32, 33</sup>, particularly Zap70 in the case of  $\zeta$  (see section 1.3.1).  $\gamma$ ,  $\delta$ , and  $\epsilon$  also contain serine / threonine residues, which can be phosphorylated by protein kinase C.

Antibody binding to CD3 results in tyrosine phosphorylation of the  $\zeta$  chain, along with calcium influx and inositol polyphosphate hydrolysis<sup>34</sup>, suggesting that CD3 occupies a position at the beginning of the activation pathway. These actions are regulated by CD4 and CD45<sup>32, 35</sup>, molecules which associate with CD3 on the cell surface<sup>36-40</sup>, as discussed below.

## 1.2.3. CD45

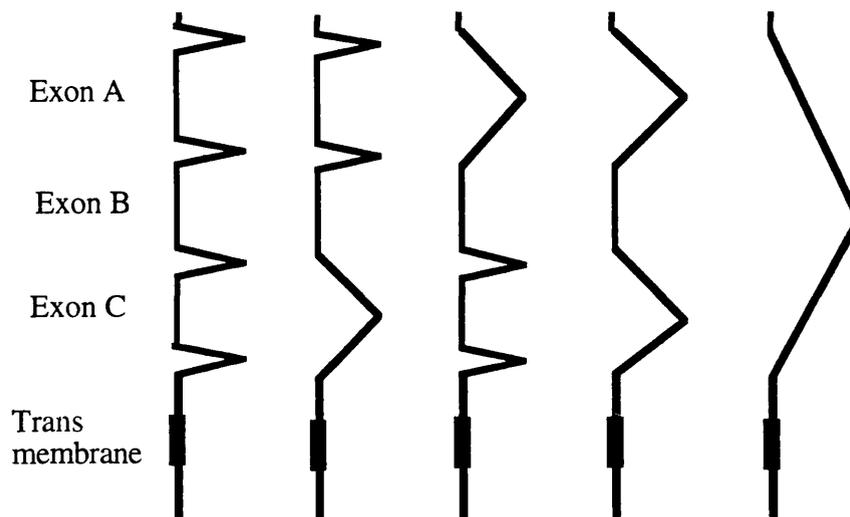
CD45 (leukocyte common antigen) is a high molecular weight (180-220kD) glycoprotein found on the surface of nucleated bone marrow derived cells. It is highly expressed, representing ~10% of surface protein and each molecule is large, on average 124nm long<sup>41</sup>. The intracellular domain is identical in all isoforms and has considerable cross species homology. This domain has tyrosine phosphatase activity<sup>42</sup> and is involved in regulation of cell activation<sup>43</sup> through its action on TKs<sup>44, 45</sup> (see sections 1.3.1 and 1.3.2.)

The extracellular domains of CD45 are affected by differential RNA splicing<sup>46</sup> and glycosylation<sup>47</sup> in different cell populations. Alternative splicing of exons 4, 5 and 6 (also known as A, B, and C) at the 5' end of the gene can produce 8 isoforms of CD45 and message for 5 of these has been detected in humans (Figure 1.1) by reverse transcriptase - polymerase chain reaction (RT - PCR). How splicing is regulated is unclear, but changes in splicing regulation take place rapidly after cell activation<sup>48</sup>. Lymphocytes from patients with haemophagocytic lymphohistiocytosis (and their families) do not appear to regulate splicing of CD45 normally and have an increased proportion of cells expressing CD45RA<sup>dim</sup> CD45RO<sup>dim</sup><sup>247</sup>.

CD45 antibodies recognise generic epitopes whilst restricted antibodies (CD45R) recognise epitopes dependent on the products of the variable exons. For example CD45RA antibodies recognise an epitope dependent on the A exon and hence can recognise 2 of the 5 detectable isoforms (see figure 1.1). The exact specificity of anti - CD45RO is not clear, although it is either a junction specific antibody or recognises carbohydrate moieties when none of the variable exons are expressed. Lack of availability of antibodies against all CD45 isoforms has hindered and confused

research in this field. Antibodies against human RB have only recently been described and those against putative RC isoforms are not well confirmed and research has therefore focused on RA and RO. Rodent work, on the other hand, has concentrated on the RB and RC isoforms.

Figure 1.1. Exon Usage In CD45 Isoforms.



	Molecular wt.	220	205	205	190	180
Reacts with	Anti - CD45RA	Y	Y	N	N	N
	Anti - CD45RB	Y	Y	Y	Y	N
	Anti - CD45RC	Y	N	Y	N	N
	Anti - CD45RO	N	N	N	N	Y

Expression of the isoforms shown in figure 1.1 is clustered; for example, one set of T cells expresses high amounts of the 220 and 205kD isoforms and lower amounts of 190 and 180 kD isoforms; these are referred to here as CD45RA+ (CD45RO-) cells. The reciprocal subset includes cells which express 180 and 190kD isoforms only and are referred to as CD45RO+ (CD45RA-)<sup>49-51</sup>. Hence CD45RA and RC are co-expressed in man, all CD45RA cells express high levels of CD45RB, whilst CD45RO+ cells express CD45RB heterogeneously<sup>52</sup>. It can be seen that this classification or nomenclature does not define isoforms absolutely; all cells in man express some CD45RB, but express variable amounts. CD45 isoform switching usually takes place on activation(1.3.3) and is associated with a variety of functional changes (1.3.1).

CD45 has a number of functions, some of which are generic and some isoform specific.

The most well characterised function is that of regulating signals through key T cell molecules such as CD4, CD3, CD2 and CD28 (see section 1.2.5 to 1.3.2). A less well defined role of CD45 is to associate in *cis* with other cells surface molecules. This tends to occur after cell activation and some authors have found a degree of isoform specificity, although consistent patterns have not emerged. For example, CD45RO has been shown to associate with CD2, CD45RA with LFA - 1 and an 190kD isoform,

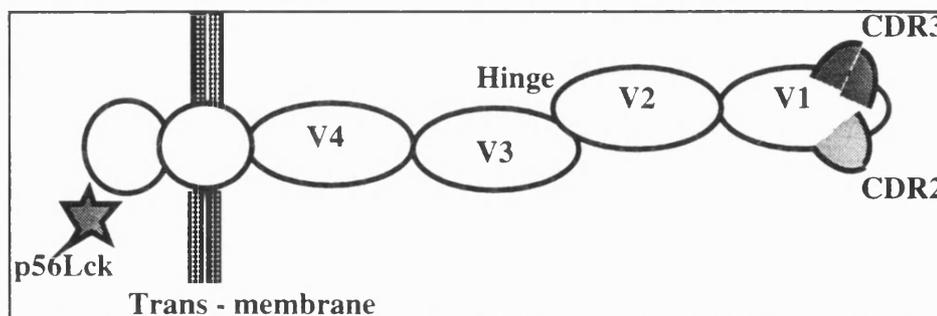
probably CD45RB, with CD4 and CD8<sup>40</sup>. CD3, CD4/8 and CD45 have been shown to co - cap, especially on CD45RO+ cells<sup>40</sup> (see section 1.2.6). An even less certain finding is the *trans* association with the B cell activation molecule CD22, ligation of which may lead to signalling in the T cell partner<sup>53</sup>. Others have claimed the CD45/CD22 interaction is non - specific<sup>54</sup>. A combination of *cis* and *trans* associations may be involved in the putative role of CD45 in the regulation of cluster stability with DCs<sup>55</sup> and B cells<sup>56</sup> (see section 1.3.2).

## 1.2.4. CD4

CD4 is a 67kD member of the immunoglobulin superfamily with a number of central roles in T cell activation. It is the ligand for MHC Class II, transmits signals to its intracellular ligand p56<sup>Lck</sup> and may be an extracellular *cis* ligand for CD45RB<sup>40</sup>. CD4 is also the receptor for HIV envelop glycoprotein, gp120<sup>57</sup>.

CD4 consists of 4 extracellular domains (V1 - cell distal - to V4) the first two of which have been studied by X ray crystallography, although analysis of the exact physical orientation of these domains proved difficult to obtain, perhaps because of its extended flexible structure (see below). Crystals confirmed the homology of V1 and V2 to an immunoglobulin  $\kappa$  light chain<sup>58, 59</sup>. Domains within V1 have sequence homology to Ig complementarity determining regions (CDRs) and the crystallography confirmed that two of these, CDR2 and CDR3, have discrete physical locations consistent with their functional differences. CDR2, for example binds HIV gp120 (see chapter 3) whilst CDR3 has an undefined role in the fusion of HIV infected cells (see chapter 4). CDR3 has also been proposed as a dimerisation site<sup>60</sup>, which is supported by the finding that the natural ligand for CD4, MHC Class II, exists as a dimer of heterodimers<sup>61</sup>. Dimerisation of CD4 may explain how sites on opposing sides of CD4 can be involved in Class II binding and also why the CDR3 region has a key role in activation<sup>62</sup>. Another possibility is that CD4 may associate extracellularly with CD45RB, with CDR3 acting as a possible link site on CD4<sup>40</sup>.

Figure 1.2. The Structure Of CD4.



The proximal part of CD4, between domains V3 and V4 may contain a hinge<sup>58, 59, 63</sup> although the physiological role of this is unclear. The cytoplasmic tail of CD4 contains a Cys-X-Cys-Pro motif which binds to p56<sup>Lck</sup> (see 1.5.5).

CD4 is a ligand for MHC Class II and during ligation signals are mediated through the associated p56<sup>Lck</sup> tyrosine kinase which modifies T cell activation (see below). CD4 ligation may also down - regulate LFA - 1 mediated adhesion<sup>64</sup>.

Two regions up - stream of the CD4 exons regulate its expression. A T cell specific enhancer switches on transcription in thymocytes, whilst a negative regulatory region switches off CD4 expression in double positive thymocytes undergoing maturation along the CD8 pathway<sup>65</sup>. Expression of CD4 and its engagement with an appropriate ligand directs thymocytes along the CD4 pathway<sup>66</sup> accompanied by development of functional T helper cell activity<sup>65</sup>. During these stages in T cell development the association between p56<sup>Lck</sup> (see below) and CD4 does not appear to be required for the development of normal T helper cell function or CD4 expression<sup>67</sup>. In mature T cells little is known about transcriptional regulation of CD4 although p56<sup>Lck</sup> is involved in the regulation of surface expression, as discussed below.

### 1.2.5. Co - stimulatory And Adhesion Molecules.

Adhesion molecules stabilise interactions between cells involved in the immune response and regulate migration. When adhesion molecules additionally transmit activation signals and augment signalling through the T cell receptor, they may be referred to as co - stimulatory molecules. Examples of these molecules are given in Table 1.1.

Table1.1 T Cell Adhesion Molecules

T cell molecule		Ligand	
Immunoglobulin Super Family	CD2	LFA - 3, CD48	Immunoglobulin
	CD28, CTLA4	B7.1, B7.2	Super Family
	ICAM - 1, 2	LFA-1	Integrin
Integrin	LFA - 1	ICAM - 1, 2	Immunoglobulin
	VLA 4	VCAM - 1	Superfamily
	VLA 5	Fibronectin	ECM
	VLA 6	Laminin	Basement membrane
	HML - 1	?mucosal ligand	
	LPAM - 1	MadCAM1	Addressin
Selectin	L selectin	MadCAM1, CD34	
Sugar	CLA	E Selectin	Selectin

CD2 (LFA - 2, T11) is a co - stimulatory molecule present on all T cells and binds to LFA - 3 (CD58) and CD48. CD2 has a major role in T cell interactions with antigen presenting cells<sup>56</sup> (APCs), which express large amounts of LFA - 3<sup>8</sup>. CD2 is linked to the TK p56<sup>Lck</sup> and to the  $\zeta$  chain of the T cell receptor and associates with CD45RO<sup>40</sup>. When CD2 is bound by specific pairs of antibodies, activation signals occur, resulting in calcium influx<sup>68</sup> cytokine secretion and proliferation<sup>69</sup>. These responses do not occur in all T cell populations<sup>69, 70</sup>, possibly because CD2 signals are regulated differently by CD45 isoforms<sup>43, 71</sup>.

The signalling role of CD28 modifies the behaviour of T cells responding to antigen presentation. CD28 is a homodimeric structure present on all CD4+ T cells and mediates its effects through a different pathway from CD3 and CD2, independent of calcium influx<sup>72</sup>. CD28 signalling may be regulated by CD45<sup>43</sup>, although CD28 binding can lead to inositol polyphosphate (IP) hydrolysis in the absence of CD45<sup>71</sup>. CD28 signalling results in increased stability of mRNA transcripts<sup>73</sup>, notably cytokine and HIV transcripts. This is brought about by the induction of a DNA binding protein, CD28RC, which binds to a  $\kappa$ B like motif<sup>74</sup>. CD28 also contributes to transcription up - regulation by induction of NF $\kappa$ B binding<sup>75</sup> (see below). Cytolytic T lymphocyte associated antigen (CTLA) - 4 is structurally related to CD28, but is only present on activated lymphocytes.

The ligands for CD28 and CTLA - 4, B7.1 (CD80) and B7.2 (B70, CD86), are present most abundantly, amongst APCs, on DCs and least on B cells<sup>9,76,421</sup>. B7 expression is up - regulated by  $\gamma$ IFN and cross linking MHC Class II<sup>30</sup>. The hierarchy of B7 expression in part reflects the ability of these cells to act as APCs<sup>8</sup> and failure of APCs to express B7 can result in anergy<sup>77</sup>, particularly of T<sub>H</sub>1 cells<sup>78</sup>. This may be the basis of some types of peripheral tolerance, in that even Class II positive non - thymic cells are unable to present successfully peptides which would otherwise be antigenic.

Intercellular adhesion molecules 1 (widespread expression), 2 (on endothelium) and 3 (leukocytes) (ICAM - 1 to 3) are the ligands for LFA - 1 (see below). The binding of LFA - 1 to lymphocyte ICAM molecules is important in heterotypic adhesion of T cells with APCs, particularly DCs<sup>8,70</sup> and spread of infectious agents<sup>79-81</sup>. Whilst ICAM - 3 is constitutively expressed, ICAM - 1 and ICAM - 2 expression is increased by cell activation, an example of "inside - out signalling".

The integrin family of proteins are non - covalently linked dimers; family members are characterised by their usage of  $\alpha$  and  $\beta$  chains. Lymphocyte function associated antigen 1 (LFA - 1), is a dimer of an  $\alpha$ 1 (CD11a) and  $\beta$ 2 chain (CD18). It associates in *cis* with CD45RA<sup>40</sup> and binds ICAM - 1, 2 and 3. LFA - 1 is involved in T cell interactions with endothelium and APCs, a role which may be differentially regulated by CD45 isoforms<sup>55,56</sup>, and also promotes HIV induced syncytium formation<sup>79, 82-85</sup>.

Although LFA - 1 expression is up - regulated after T cell activation<sup>86</sup>, a much faster and more effective process is the increase in LFA - 1 affinity seen after signalling through the TcR<sup>87</sup> and CD3<sup>88</sup> or exposure to soluble factors released by endothelium<sup>89</sup>.

This 200 fold<sup>90</sup> increase in LFA - 1 affinity lasts less than an hour, requires protein kinase C (PKC) activity<sup>88</sup> and appears to be regulated by changes in the phosphorylation of the intracytoplasmic part of the molecule or increased association with the cytoskeleton<sup>91, 92</sup> and exposure of special epitopes on the extracellular part of the molecule<sup>90</sup>. Binding of antibodies to CD45 also increases LFA - 1 affinity, without upregulating LFA 1 expression, but does not use PKC activation<sup>91</sup>. These processes are referred to as “inside - out - signalling”.

Ligation of CD4, on the other hand, reduces LFA - 1 mediated adhesion<sup>56,93</sup>, a process requiring p56<sup>Lck</sup> activity<sup>64</sup>. Dissociation of LFA - 1/ligand is required as cells move across endothelium and when APC/T cell clusters dissociate. A synthesis of these findings is that CD4 ligation by MHC Class II molecules not presenting an appropriate antigen will result in rapid disaggregation of T cell/APC clusters; when the TcR is stimulated, the negative CD4 signal is abrogated and the clusters remain intact, allowing T cell activation to take place<sup>64</sup>.

LFA - 1 also transmits “outside - in” calcium signals after ligation and its ligation can act as a co - stimulus for cytokine secretion<sup>94</sup>.

Another integrin, very late antigen 4 (VLA4:  $\alpha 4$  chain (CD49) and  $\beta 1$  chain (CD29) binds vascular cell adhesion molecule (VCAM) 1 on cells or fibronectin in matrix<sup>95</sup> and transmits activation signals, which are modulated by CD45<sup>96</sup>. VLA4 may confer homing specificity for migration into skin<sup>90</sup>. CD29 has been used as a marker of memory T cells<sup>97</sup>, prior to the discovery of antibodies against the CD45RO isoform, although the correlation between high CD45RO and CD29 expression is not absolute. The other VLA molecules, VLA 5 and VLA 6, also contain the  $\beta 1$  integrin chain (CD29) and bind to fibronectin and laminin respectively<sup>95</sup>. VLA molecules require T cell activation to enhance binding<sup>95</sup>.

Two additional integrins confer homing specificity to specialised populations of lymphocytes; LPAM - 1 ( $\alpha 4\beta 7$  integrin) and HML-1 ( $\alpha E\beta 7$  integrin) bind MADCAM - 1 (mucosal vascular addressin cell adhesion molecule - 1) and to an unknown mucosal ligand, respectively<sup>90</sup>. These are discussed in section 1.4.5.

L selectin also binds the addressin MADCAM - 1. MADCAM - 1, which contains sugar residues related to Sialyl Lewis<sup>X</sup>, is expressed on mesenteric lymph node high endothelial venules and gut mucosa endothelium and confers homing on cells expressing L selectin. E selectin, expressed predominantly on skin endothelium binds to the cutaneous lymphocyte associated antigen (CLA). CLA expressed on subsets of T cells, is another Sialyl Lewis<sup>X</sup> containing molecule.

These molecules and their role in cellular homing are discussed further in section 1.4.5.

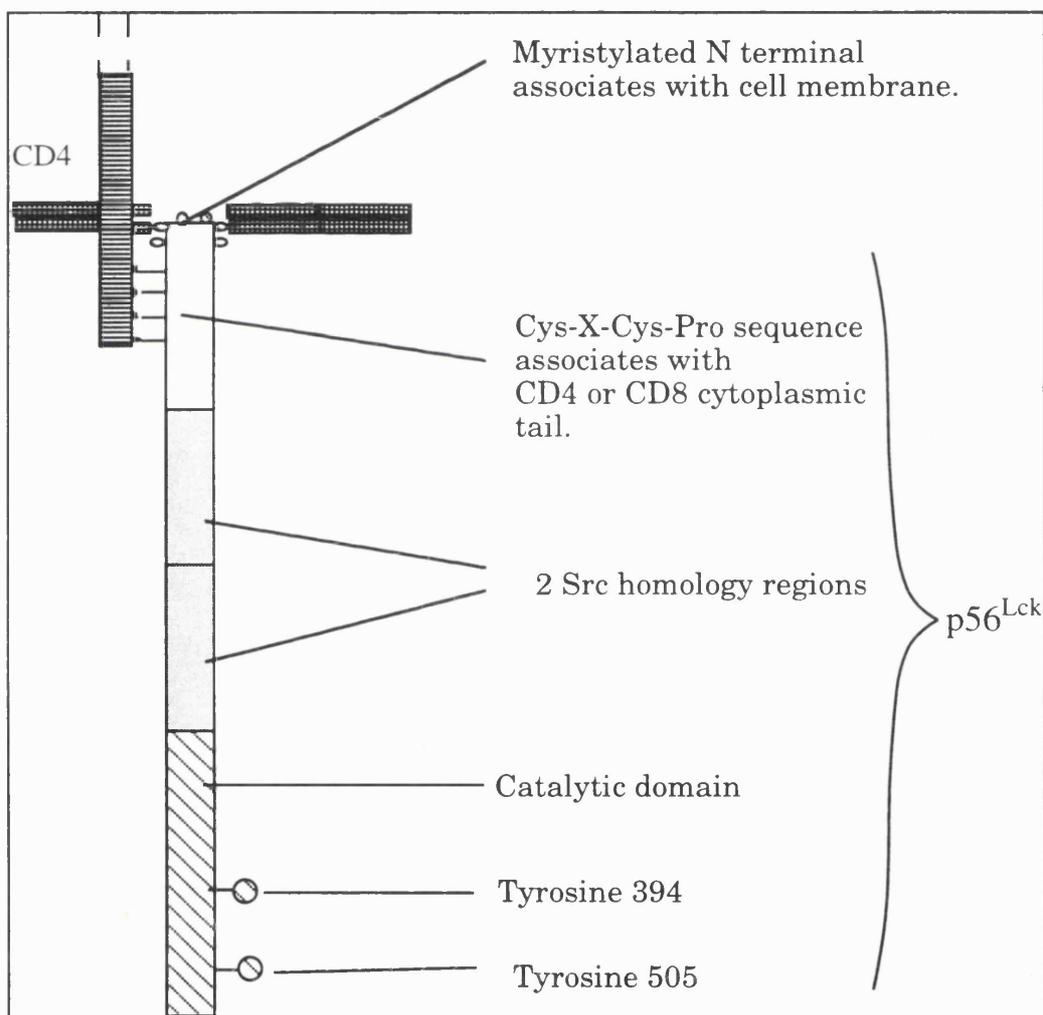
### 1.2.6. p56<sup>Lck</sup>.

The finding that cross linking of CD4 leads to protein phosphorylation suggested that CD4 is associated with a protein kinase (reviewed in<sup>35</sup>). Since cross

linking of CD4 or CD8, but not other surface molecules, leads to increased activity of p56<sup>Lck</sup>, a member of the *Src* oncogene family, it was concluded that this molecule was the relevant kinase<sup>98</sup>. In common with other members of the *Src* oncogene family, p56 has specific constitutive molecular associations and two regulated functions, carboxy terminal TK activity and SH2 domain phosphotyrosine binding activity.

The N terminus of p56<sup>Lck</sup> and the cytoplasmic tails of CD4 and CD8 all contain Cys-X-Cys-Pro motifs. The motifs in CD4/8 co - ordinate with those in p56<sup>Lck</sup>, possibly with an intervening metal ion<sup>99</sup>. These motifs do not occur in other *Src* family members and confer the specificity of p56<sup>Lck</sup> for CD4/8. Additionally, the N terminus of p56<sup>Lck</sup> is myristylated to maintain its position at the inside face of the cell membrane.

Figure 1.3. The Structure Of p56<sup>Lck</sup>.



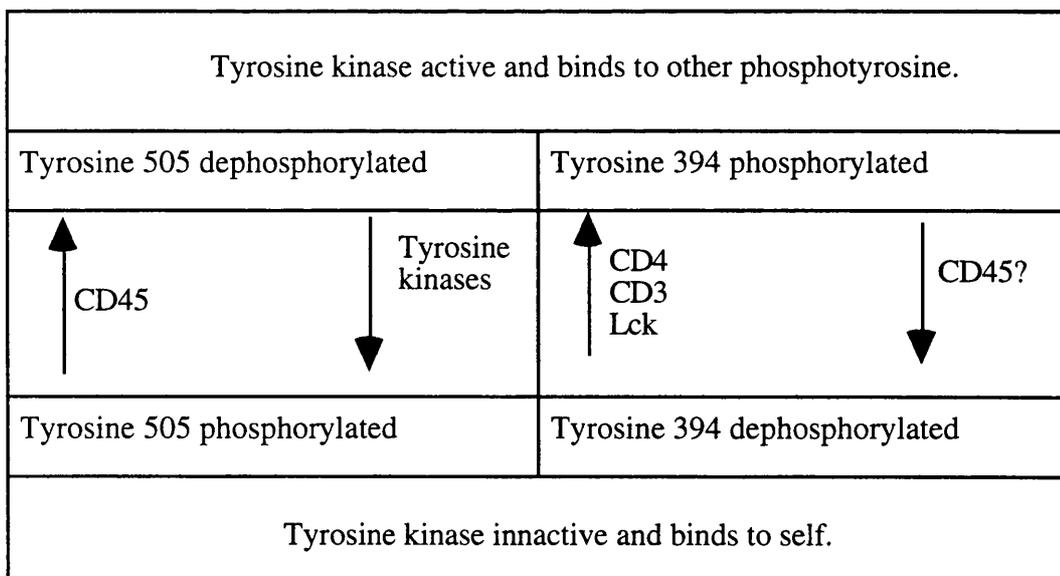
The kinase activity of p56<sup>Lck</sup> is up - regulated when the tyrosine residue at 394 is phosphorylated and that at 505 dephosphorylated<sup>100, 101</sup>. CD4 cross linking results in phosphorylation of Tyr394 and subsequent switching on of p56<sup>Lck</sup> kinase activity<sup>98, 102</sup>. Phosphorylation of p56<sup>Lck</sup> by CD4 binding is reduced when CD45 is simultaneously cross linked (unpublished data in <sup>44</sup>). CD3 cross linking alone up - regulates p56<sup>Lck</sup> kinase activity when CD45 is present<sup>102</sup> and boosts the signal provided

by CD4 cross linking<sup>103</sup>. *In vitro* CD45 dephosphorylates the negative regulatory tyrosine at position 505 to increase p56<sup>Lck</sup> kinase activity<sup>45, 104</sup>. Pairs of antibodies binding to CD45 induce activation of p56<sup>Lck</sup> and facilitate its activation by CD2<sup>100</sup>, possibly because CD45 requires dimerization or phosphorylation before it can activate p56<sup>Lck</sup>. Consequently, when CD45 is absent, p56<sup>Lck</sup> activity is reduced<sup>45</sup> and signal transmission through CD3 is impaired<sup>71, 101</sup>. In other T cell lines CD45 is not required for p56<sup>Lck</sup> activity<sup>101</sup> and Fyn rather than p56<sup>Lck</sup> has been implicated as the CD45 regulated TK<sup>105</sup>. This has led to the idea that p56<sup>Lck</sup> may have more than one role and that in its function as a kinase it may be replaced by p59<sup>Fyn</sup>.

A second role for p56<sup>Lck</sup> is in co - ordinating, non - covalently, different molecules in the T cell signalling system. Although p56<sup>Lck</sup> binds to CD4 or CD8 in the specific fashion described above, *Src* homology regions(SH2) within p56<sup>Lck</sup> can also bind to a number of tyrosine phosphorylated proteins. The p56<sup>Lck</sup> SH2 domain can bind to its own carboxy terminal, the carboxy terminal of another p56<sup>Lck</sup> molecule or other *Src* family members such as Fyn<sup>102</sup>. p56<sup>Lck</sup> also binds to the ζ chain, but only after it has been phosphorylated<sup>106, 107</sup>. This phosphotyrosine binding function of p56<sup>Lck</sup> may be more important than the kinase function since its ablation, but not that of the kinase domain, can inhibit T cell clone function<sup>107</sup>.

Whether p56<sup>Lck</sup> binds intramolecularly or to separate phosphotyrosine motifs it is regulated by the same mechanisms as kinase activity; in this case dephosphorylation of tyrosine 505 by CD45 or phosphorylation of 394 by CD4 or CD3 ligation favours binding of exogenous motifs<sup>102</sup>. These regulatory mechanisms are summarised below.

Figure 1.4. Regulation Of p56<sup>Lck</sup>.



Hence binding of CD4, especially when CD3 is also ligated, and when CD45 is also present leads to optimal p56<sup>Lck</sup> kinase activity. This parallels the physiological situation in which CD4 is bound to Class II MHC in the locality of antigen binding to the T cell receptor, in the presence of CD45.

The result of these molecular interactions is T cell hyper - responsiveness. For example, a mouse T cell hybridoma, deficient in CD4 expression, was transfected with a constitutively activated p56<sup>Lck</sup>, with phenylalanine at 505 instead of tyrosine. In response to its antigen or anti - CD3 this hybridoma produced more IL2 and more protein phosphorylation than the parent line<sup>108</sup>.

### 1.2.7. p56<sup>Lck</sup> Interactions.

CD4 physically associates with CD3/TcR at the T cell surface, particularly after antigen specific<sup>36, 37</sup> or polyclonal<sup>38, 39</sup> stimulation. This association requires CD4 to have an intact CD4 cytoplasmic tail<sup>38</sup> and p56<sup>Lck</sup> association motif<sup>109</sup>. The molecular basis of this interaction is likely to be that T cell activation switches on p56<sup>Lck</sup> kinase and phosphotyrosine binding activities, target sites on CD3 sub - units become phosphorylated and subsequently bound by p56<sup>Lck</sup>.

CD45 is required to facilitate p56<sup>Lck</sup> phosphotyrosine binding activity (see section 1.2.5), and hence CD4/CD3 association and also appears to be integrated into these complexes itself. In the mouse CD45RB+ but not CD45RB- isoforms co - cluster with CD4 and CD3<sup>39</sup>, although in another study there was no evidence of co - clustering of CD45<sup>38</sup>. In human T cells, CD45RO associates with CD2 and may modulate signalling through this molecule<sup>110</sup>, while CD45RA associates with LFA - 1. CD3, CD4/8 and CD45 isoforms co - cap with a 190kD CD45 isoform<sup>40</sup>. Since this occurred more in CD45RO+ cells, this isoform is probably CD45RB, which is the only 190kD isoform known to be highly expressed in this population<sup>49, 50</sup>. A CD45RB epitope also transmits a positive signal when ligated, suggesting a specific cell surface ligand<sup>47</sup>.

Less direct evidence for the extracellular association between CD45 and CD4 comes from the observation that, in mice, anti - CD45 antibodies only modulate CD3 signals in CD4+ and not CD8+ cells, even though both are equipped with p56<sup>Lck</sup> and fyn<sup>111</sup>.

CD4 undergoes constant recycling via the clathrin coated pit pathway<sup>112</sup>. In quiescent T cell lines this process is tightly regulated and CD4 expression remains stable with 5% of CD4 intracellular at any one time. When T cells are transfected with a CD4 molecule without a cytoplasmic tail or non - T cells (which do not have p56<sup>Lck</sup>) are transfected with intact CD4, recycling is faster and a higher proportion of CD4 remains intracellular<sup>113</sup>. Transfection of p56<sup>Lck</sup> into non - T cells slows down recycling of co - transfected intact CD4<sup>114</sup>, suggesting that p56<sup>Lck</sup> delays CD4 entry into the endocytic pathway.

The dynamic equilibrium of CD4 expression can be disturbed in a number of ways: PKC activity results in phosphorylation of CD4 and its subsequent down - regulation<sup>115</sup>. This can occur in CD4 transfected HeLa cells, in which p56<sup>Lck</sup> is absent<sup>116</sup>, but not when the cytoplasmic tail of CD4 is absent<sup>117</sup>. CD4 down - regulation is abrogated when serine residues in the cytoplasmic tail, constituting a potential PKC substrate site, are deleted or when the PKC antagonist H7 is added<sup>117</sup>. Transfection of p56<sup>Lck</sup> into a monocytoid line partially protects against PMA induced down - regulation of CD4<sup>118</sup>, although TK inhibitors do not affect this p56<sup>Lck</sup> mediated rescue<sup>118</sup>. Hence PKC activity on the CD4 cytoplasmic tail down - regulates CD4 expression; Lck can abrogate this, independently of its TK activity.

Alternatively, binding of anti - CD4 antibody (or gp120) down - regulates CD4. This appears to be coincident with increased phosphorylation and kinase activity of p56<sup>Lck</sup> along with dissociation from CD4<sup>119, 120</sup>. The dissociated p56<sup>Lck</sup> is then sequestered from the detergent soluble fraction, presumably by associating with the cytoskeleton<sup>103</sup>. Inhibition of TK with either herbimycin or genistein augments anti - CD4 mediated down - regulation of CD4<sup>121</sup>. When CD3 and CD4 are cross linked simultaneously, p56<sup>Lck</sup> is activated but does not dissociate from CD4<sup>103</sup>.

In addition to masking epitopes relevant for gp120 binding<sup>122, 123</sup>, dextran sulphate and gangliosides down - regulate CD4. In the case of gangliosides, the mechanism for this down - regulation is independent of the cytoplasmic tail of CD4 and so does not involve p56<sup>Lck</sup><sup>123</sup>.

In summary, ligation of CD4 or phosphorylation of its cytoplasmic tail lead to CD4 down - regulation. The presence of p56<sup>Lck</sup> abrogates this, in the case of CD4 ligation, through its kinase activity. Ligation of CD4 by MHC Class II helps form T cells/APC clusters. The physiological correlate of CD4/p56<sup>Lck</sup> dissociation and CD4 down - regulation may be that after the initial stages of antigen presentation and T cell activation, the T cell/APC cluster can separate<sup>124</sup>. Another correlate of cross linking CD4 alone may be uncoupling of CD3, leading to a state of partial activation and priming of the cell for apoptosis<sup>125</sup>.

## 1.3 T Cell Activation.

### 1.3.1 Protein Tyrosine Kinase Activation.

Protein tyrosine phosphorylation is one of the earliest steps in T cell activation and is seen within seconds of T cell stimulation. TK inhibitors block early steps, such as calcium influx and inositol polyphosphate hydrolysis (reviewed in <sup>31, 126</sup>). A major problem has been identifying which TKs could be responsible for linking the T cell receptor to the activation cascade. With analogous receptors, such as the epidermal growth factor and insulin receptors, kinase activity is part of the receptor molecule itself, but none of the CD3 subunits possess intrinsic TK activity. Three TKs are important candidates for bridging the gap between CD3 and later signalling events. p56<sup>Lck</sup> is a likely candidate since it has important and regulatable roles in tyrosine phosphorylation and phosphotyrosine binding, which have been discussed above. PLC $\gamma$ 1 is a substrate for p56<sup>Lck</sup> <sup>105</sup> and is the only enzyme involved in signalling in which constitutive activity has been linked to T cell hyper - responsiveness<sup>108</sup>.

On the other hand, the *Src* family member p59<sup>Fyn</sup> co - precipitates with CD3, although its kinase activity and association with phosphotyrosine have not been clearly demonstrated to increase after stimulation of mature T cells<sup>126</sup>. p59<sup>Fyn</sup> is a substrate for CD45<sup>105</sup>.

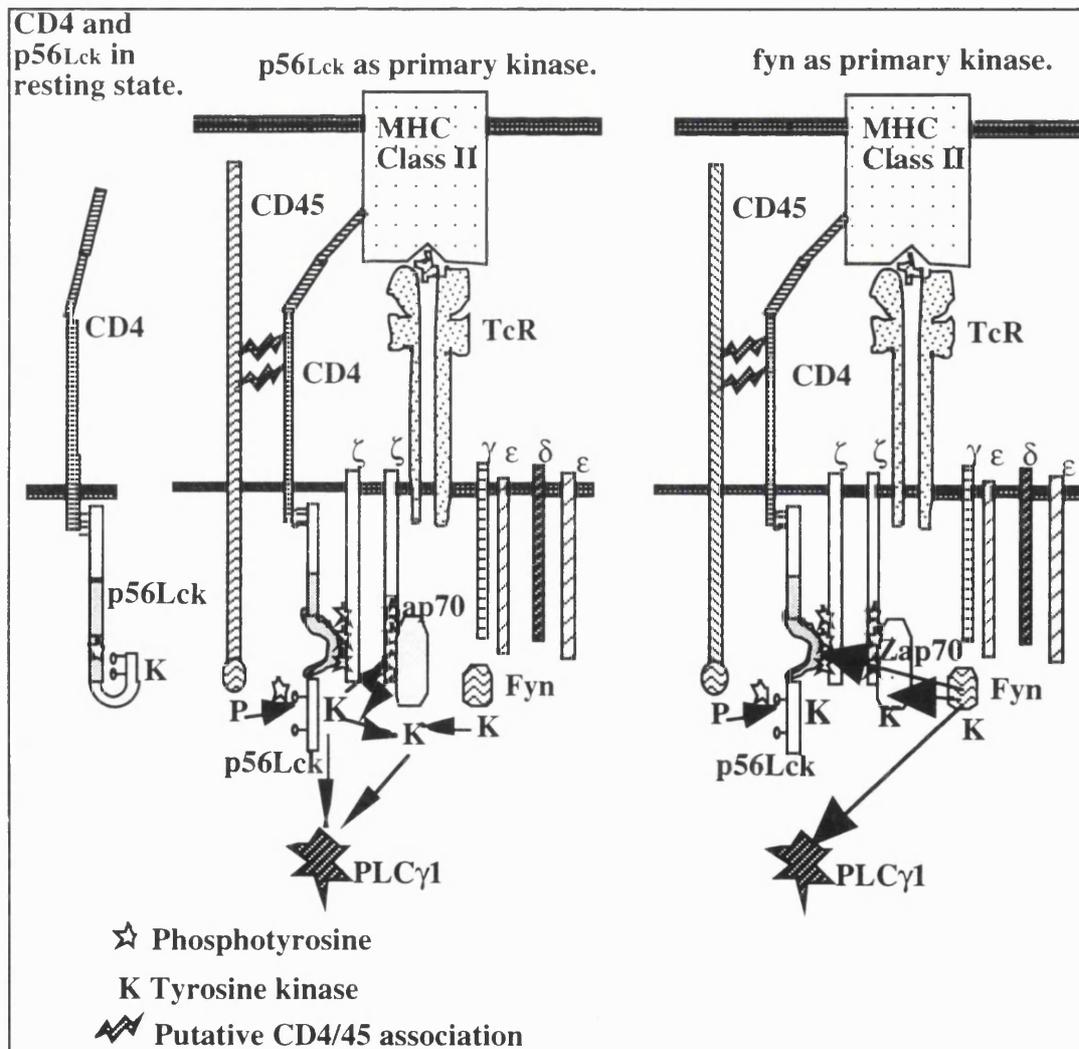
$\zeta$  associated peptide (Zap 70) is a TK which does not share all features of *Src* family members; it does not have a myristylation site and has no COOH terminal

phosphorylation/regulation site. Zap 70 can only associate with tyrosine residues on CD3 sub - units once they have been phosphorylated, possibly by p56<sup>Lck</sup>, and itself becomes a target for p56<sup>Lck</sup> or p59<sup>Fyn</sup> (discussed in <sup>33</sup>).

In the complex model that emerges, p56<sup>Lck</sup> appears to have a key role in early tyrosine phosphorylation. It is uncertain whether this role hinges on the kinase ability of p56<sup>Lck</sup> or its ability to act as an adaptor between different molecules. In one model p56<sup>Lck</sup> first phosphorylates the  $\epsilon$  and  $\zeta$  chains<sup>98</sup>, by acting on the tyrosine residues in the ARAMs<sup>33</sup>. Zap70 can then associate with these sub units and becomes a substrate for p56<sup>Lck</sup>. Simultaneously, p56<sup>Lck</sup> activates PLC $\gamma$ 1<sup>105</sup> and other regulatory proteins, such as microtubule associated protein (MAP) family members<sup>127</sup>.

Alternatively, p59<sup>Fyn</sup> initiates the activation cascade, activates p56<sup>Lck</sup> hence intercalating it between CD4 and CD3. In this model the role of p59<sup>Fyn</sup> is predominantly as a kinase and that of p56<sup>Lck</sup> as an intermolecular bridge. This is illustrated below in figure 1.4

Figure 1.5. p56<sup>Lck</sup> and interactions.



### 1.3.2. Inositol Polyphosphate, Calcium Influx And Protein Kinase C In T Cell Activation.

Activation of phospholipase C  $\gamma$ 1 (PLC $\gamma$ 1) by a TK, most likely p56<sup>Lck</sup> or p59<sup>Fyn</sup>, results in the rapid hydrolysis of phosphatidylinositol 4,5 biphosphate (PIP<sub>2</sub>) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP<sub>3</sub>). Increased intracellular IP<sub>3</sub> concentration mediates an influx of calcium ions which, together with DAG, activate PKC. PKC directly phosphorylates a number of transcription factors, as discussed in the next section. The influx of calcium ions also activates calcineurin, which in turn is responsible for the nuclear translocation of the nuclear factor of activated T cells 1 (NFAT1). PKC activation increases adhesion through LFA - 1/ICAM - 1<sup>91</sup>, whilst TK activation may do so through other integrins<sup>128</sup>.

Fluorescent dyes which change their emission spectra at different concentrations of intracellular calcium have facilitated the investigation of the molecular basis of early activation in different T cell populations<sup>129</sup>. Anti - CD3 was the first ligand found to result in calcium influx in T cells<sup>129</sup>. Subsequent experiments showed that calcium signals are also elicited by antibodies against a variety of other T cell surface molecules, including CD2, CD4, and CD8. However, only calcium signals mediated by anti - CD3 do not require cross linking and stimulate cell proliferation<sup>68</sup>.

Binding of both intact anti - CD4 antibody and F(ab') fragment can produce calcium influx. Anti - CD4 antibody can inhibit subsequent CD3 induced signals, whilst F(ab') fragment cannot<sup>130</sup>. Similarly, heteroconjugate binding of CD3 and CD4 together augments the CD3 induced signal, whilst separate CD3 and CD4 signalling diminishes it<sup>35, 36</sup>. These data suggest that anti - CD4 antibody inhibits CD3 mediated signals by sterically preventing CD4 from fulfilling its co - receptor role<sup>131</sup> or by sequestering p56<sup>Lck</sup> in the absence of a concurrent CD3 signal.

Since CD45 regulates the TK and phosphotyrosine binding activities of p56<sup>Lck</sup>, which in turn links CD3 to proximal signalling mechanisms, it would be expected that CD45 can also modify CD3 mediated calcium signals. Initial experiments showed that cross linking anti - CD3 and anti - CD45 diminished calcium responses<sup>43</sup>. Cross linking CD4 and CD45 in the same way augmented responses. This reduction in CD3 mediated signalling can alternatively be read out as proliferation<sup>43</sup> or cytokine secretion<sup>94</sup> and was explained by an inhibitory effect of CD45 on early T cell signalling<sup>132</sup>. This is not easy to reconcile with the finding by several groups that CD45- T cell lines have an impairment in CD3 mediated calcium signalling and IP hydrolysis, which can be restored with CD45 transfection<sup>101, 105, 133</sup>. Furthermore, CD3 and CD45 have been shown to associate, as described above, in physiological activation conditions<sup>39</sup>.

An alternative explanation is that CD3 dimerization is required for activation and when CD3 and CD4 or CD45 are cross - linked by antibodies, CD3 cannot dimerize<sup>134</sup>. Additionally, CD45 isoforms interact extracellularly with other surface molecules involved in signalling<sup>40</sup>; anti - CD45 antibodies may prevent these interactions. Other anti - CD45 antibodies have a positive effect<sup>47</sup> on CD3 signalling

and either bind epitopes, present only on CD45RO+ cells, which either normally have negative effects on signalling or induce a positive intracellular signal through CD45.

As explained in section 1.3.1, p56<sup>Lck</sup> or p59<sup>Fyn</sup> probably act as intermolecular bridges between the components of the T cell signalling mechanism. P59<sup>Fyn</sup> is favoured from experiments on human cells showing decreased responsiveness to anti - CD3 does not correlate with decreased p56<sup>Lck</sup> activity in CD45 negative cells<sup>101, 105</sup>, signalling through CD3 was restored by cross linking CD3 and CD4<sup>101</sup> emphasising the alternative role of p56<sup>Lck</sup>, as an intermolecular adapter, which may be bypassed by surface molecule cross linking. In this case the positive effects of p56<sup>Lck</sup> on signalling<sup>108</sup> may reflect more the juxtaposition of molecules by p56<sup>Lck</sup> than its kinase activity (see figure 1.4 above).

Although TK activity is the same in CD45RA+ and RO+ cells after CD3 stimulation<sup>135</sup>, subsequent calcium influx is greater in CD45RO+ populations<sup>136, 137</sup>; whether interactions between CD3, CD4 and CD45, both intracellular and on the cell surface, contribute to this is unclear. CD3 and CD4 cluster preferentially with CD45RO rather than CD45RA<sup>40</sup> and low molecular weight CD45RB associates with CD4<sup>40, 47</sup>. Clearer evidence of the importance of surface clustering comes from stimulation through CD2; calcium influx is greater in CD45RO+ cells<sup>137</sup> and it is known that CD2 associates preferentially with CD45RO<sup>40</sup>.

In conclusion, early signals from CD3 are transmitted optimally when CD3, CD4 and CD45 are clustered. CD3 and CD4 may associate through interactions between p56<sup>Lck</sup> and the  $\zeta$  chain of CD3, whilst CD4 and CD45 may associate extracellularly, a process which may be more effective with isoforms of CD45 expressed on CD45RO+ cells.

### 1.3.3. Tumour Necrosis Factor And T Lymphocytes.

Tumour necrosis factor (TNF) is one of several cytokines capable of modifying the response of T cells to signals transmitted by the TcR. TNF has also been thought to have a special role in the pathogenesis of AIDS<sup>138</sup>.

TNF  $\alpha$  and  $\beta$  are secreted by monocytes and T lymphocytes, respectively. TNF  $\alpha$  and  $\beta$  mediate pleiomorphic effects on a wide variety of cell types ( reviewed in <sup>139</sup>).

Although neither TNF  $\alpha$  nor  $\beta$  have any effect on resting T lymphocytes (see below), in polyclonally activated T cells and T cell lines they both enhance proliferation<sup>140-143</sup> by increasing sensitivity to limiting concentrations of IL2<sup>140, 144</sup>. This is achieved by transcriptionally increasing expression of the alpha chain of the interleukin 2 receptor (IL2R $\alpha$ )<sup>140, 144-146</sup>, although TNF has no effect on IL2 secretion<sup>144, 146</sup>. A similar mechanism increases T cell NK activity<sup>94, 147-149</sup>. Paradoxically, high concentrations of TNF do not have these effects<sup>142, 147, 150, 151</sup>. TNF also increases MHC Class I<sup>152</sup> and Class II<sup>140</sup> expression and  $\gamma$ IFN secretion<sup>140</sup>. Finally, TNF is able to induce apoptosis in a number of vulnerable T cell lines<sup>153, 154</sup> and in T cells normally resistant to this effect when they are infected with HIV<sup>155-157</sup>.

In addition to these direct effects on T cells, TNF affects other cells with which T cells interact in the immune response. For example, TNF enhances lymphocyte migration through endothelium by stimulating chemotactic IL8 release<sup>158</sup>) and increasing endothelium adhesion molecule expression<sup>159</sup>. Monocytes secrete IL1 and prostaglandin<sup>160</sup> in response to TNF although TNF has monocyte mediated immunosuppressive effects when given at high concentration *in vivo*<sup>161</sup>. TNF promotes preferential development of DCs from CD34+ marrow cells<sup>160</sup>.

The effects of TNF effects on T cells are potentiated by preactivation, which increases TNF binding<sup>140</sup>. This occurs after polyclonal activation with PHA<sup>162</sup>, anti - CD3<sup>152</sup>, IL1, IL4, IL6<sup>163</sup>,  $\gamma$ IFN<sup>164</sup>, phosphodiesterase inhibitor<sup>165</sup> and infection of cells with HIV<sup>166</sup>. Phorbol esters cause a transient decrease followed by a sustained increase in TNF binding<sup>167</sup>.

Two TNF receptors have been characterised<sup>168</sup> and belong to a family of 10 other receptors, including CD40 and Fas (see below), and 8 ligands<sup>169</sup>. A 55kD and 75kD TNF receptor have been identified in man and react with monoclonal antibodies htr-9 and utr-1, respectively<sup>170</sup>. P55 binds xenogeneic TNF whilst p75 is species specific<sup>171, 172</sup>. Neither receptor preferentially binds TNF $\alpha$  or TNF $\beta$ <sup>173</sup>.

Limited data on fresh T cells shows negligible expression at rest with up - regulation of the p75 receptor after stimulation with anti CD3, IL2<sup>167</sup> or phorbol ester<sup>174</sup>. Expression of p55, at least on monocytes, is post transcriptionally up - regulated after exposure to phorbol ester<sup>175</sup>.

In some situations p55 and p75 appear to be regulated separately on the same cells<sup>174</sup>. Hence, p75 only is up - regulated by cAMP agonists<sup>176</sup>, IL2<sup>167</sup> or pairs of CD2 antibodies<sup>177</sup>. There are also differences in tissue distribution; p55 is expressed on epithelial cells whilst p75 predominates on lymphoid cells<sup>167, 170</sup>. Similarly, studies on lymph node biopsy material suggest up - regulation of the p75 TNFR in the T cell area from inflamed lymph nodes, concomitantly with increased IL2R $\alpha$  expression whilst p55 is expressed mostly on follicular DCs<sup>178</sup>.

TNF $\alpha$  is secreted into the cell membrane and so can form part of the intercellular co - stimulatory molecule complex, TNF $\beta$  is secreted from the cell<sup>169</sup>. TNF forms tetramers in solution<sup>179</sup>, as does TNFR p55 on the cell surface. Hence receptor / ligand clusters form on exposure<sup>180</sup> and may be enough to induce signals<sup>169, 181</sup>.

Intense stimulation of T cells results in proteolysis and release of a soluble component of the TNF receptor of TNF<sup>167, 175, 182</sup>. Soluble TNFR is thus found in the blood and urine of patients with cancer<sup>175</sup> and AIDS<sup>183</sup>. *In vitro* data have shown soluble TNFR reduce initial available concentrations of TNF, but also prevent degradation, resulting in more available TNF long term<sup>184</sup>. This may be a negative feedback mechanism to prevent surges of high concentration TNF after stimulation<sup>167</sup>. sTNFR also confounds TNF bio and immuno - assays and contributes to conflicting data on TNF levels in the blood of patients with AIDS.

Anti - p75 ligation results in T cell proliferation<sup>171</sup> and NF $\kappa$ B induction<sup>176</sup>. P55 ligation has more pleiomorphic effects and results in gene induction<sup>171</sup> and either

proliferation or apoptosis, depending on the cell type and factors such as the presence of viral infection<sup>155, 156</sup>. P55 is the key receptor mediating the immune response to intracellular organisms and systemic toxicity to high concentrations of TNF<sup>185</sup>. Binding of TNF to p55 and p75 results in NF- $\kappa$ B induction even at low receptor occupancy<sup>173</sup>.

Although TNFR p55 and p75 do not associate on the cell surface<sup>176</sup> they appear to interact. The  $K_D$  values of p55 and p75 for TNF are 0.5 and 0.1 nM, respectively<sup>168</sup>, but binding of TNF to p75 promotes binding to p55. At low concentrations TNF binds to the higher affinity p75 receptor and proliferation is the dominant response; there is then up - regulation of IL2 $\alpha$  (induced by TNF) and TNFR p75 (induced by IL2)<sup>167</sup>. At higher concentrations TNF binds to p55, facilitated by p75, and either cytotoxicity or proliferation, depending on the cell type, occur<sup>168</sup>.

TNF receptor occupancy leads to the generation of a number of second messengers. For example, induction of both cAMP and PKC are required for TNF to induce IL6<sup>186, 187</sup>, whilst PKC alone induces the Fos - Jun transcription factor, AP1<sup>188</sup>. TNF also mediates changes in the redox potential of the cell and induces expression of the protective enzyme manganous superoxide dismutase (MnSOD), suggesting TNF uses reactive oxygen intermediaries (ROIs) as a unique (in T cells) third set of messenger molecules<sup>189</sup>. The outcome of ROI induction depends on the cell type. In cells unable to induce MnSOD ROIs cause mitochondrial damage and apoptosis ensues<sup>190</sup>. In cells resistant to its cytotoxic effects, TNF up - regulates the synthesis of MnSOD and directs the ROIs into a pathway where they safely deplete cellular glutathione<sup>191</sup> and in doing so induce NF $\kappa$ B<sup>192</sup> (see below).

A much less well characterised TNF family ligand/receptor pair has recently been identified (reviewed in <sup>193</sup>). The ligand, LT $\alpha_2\beta$ , forms heterotetramers with TNF $\beta$  and may be involved in cell surface anchoring of this cytokine. Deletion of the receptor for LT $\alpha_2\beta$  results in poor lymph node formation, suggesting this ligand pair may be required for homing.

Fas is a TNFR family member with extracellular homology to both TNF receptors, nerve growth factor receptor, CD27 and CD40 and has intracellular homology with CD40 and TNFR p55<sup>194</sup>. TNFR and Fas are down - regulated by anti Fas and TNF, respectively<sup>195</sup> and Fas is up - regulated by  $\gamma$ IFN<sup>194</sup>.

The ligand for Fas is cloned but uncharacterised, but anti - Fas binding mediates pleiomorphic cellular events. Binding of anti - Fas to resting T cells results in up - regulation of proliferation (IL2 independent), IL2R $\alpha$  and adhesion molecule expression. Anti - Fas induces apoptosis in T cell clones<sup>196</sup> and activated T cells<sup>197</sup>. A shared homology in the intracellular part of Fas and p55 is involved in cytotoxicity, but existing evidence suggests the two molecules do not exert their effects in the same way<sup>198</sup>. For example, although they express both Fas and TNFR p55, T cell lines may undergo apoptosis only when treated with anti - Fas<sup>198</sup>. Like TNF, anti - Fas selectively kills HIV infected cells and Fas is up - regulated by HIV infection<sup>199</sup>.

*C - myc* is an oncogene with a key role in these pathways; when *C - myc* is activated, apoptosis ensues, unless *bcl2* is also active, in which case proliferation<sup>200</sup> or

even uncontrolled growth<sup>201</sup> occurs. The B cell lymphoma oncogene *bcl2* acts against the apoptosis inducing effects of Fas and TNF<sup>198, 200</sup> and has clonogenic effects, causing autoimmunity in transgenic mice<sup>202</sup>. Autoimmunity and lymphoproliferation are also seen in *lpr* mice with dysfunctional Fas<sup>203</sup>, *gld* mice with abnormal Fas ligand genes<sup>204</sup> and mice with “knocked out” p55<sup>169</sup>. Hence *c - myc*, Fas and p55 can induce apoptosis or proliferation; the latter is favoured by the presence of *bcl2*. These effects exert restraining activity on the immune response as discussed in section 1.4.2. Other unidentified molecules must be involved, since T cells from the mouse strains mentioned are still able to undergo apoptosis when exposed to superantigen or steroid<sup>204</sup>.

*Fas* is up - regulated and *bcl2* down - regulated on primed T cells expressing CD45RO, a population predisposed to apoptosis on deprivation of IL2<sup>52</sup> (see below). This population of cells requires close regulation, since their uncontrolled expansion could be a cause of autoreactivity<sup>124</sup>.

### 1.3.4 T Cell Transcription Factors.

T lymphocyte activation is dependent on a complex series of events, culminating in the secretion of IL2 and up - regulation of IL2R $\alpha$ . These pivotal events commit T cells to proliferation and require induction of transcription factors NF- $\kappa$ B, NFAT1<sup>205, 206</sup> and AP1<sup>206</sup>. Enhancers are regions of genes which interact with cellular transcription factors to augment transcription. They may be remote from the transcription initiation site, able to act regardless of orientation and are often multimeric. The  $\kappa$ B sites are archetypal enhancers and are necessary for the transcription of IL2, IL2R $\alpha$  and HIV.

#### 1.3.4.1. Transcriptional regulation through the $\kappa$ B site.

Transcriptional regulation through the  $\kappa$ B site involves three sets of proteins; the NF- $\kappa$ B family members p50 and p65, their inhibitor I $\kappa$ B and proteins containing zinc fingers.

NF- $\kappa$ B was originally identified as a nuclear factor in activated B cells, which bound to the enhancer of immunoglobulin  $\kappa$  light chains<sup>207</sup>. Subsequently, NF- $\kappa$ B was found to act as a T cell transcription factor for 3 classes of genes; 1) immunomodulatory cytokines, IL2<sup>208, 209</sup>, IL6<sup>210</sup>, GM-CSF<sup>211</sup>, TNF $\beta$ <sup>212</sup>, TNF $\alpha$ <sup>213</sup>,  $\beta$  interferon<sup>214</sup>, 2) immunoregulatory cell surface molecules IL2R $\alpha$ <sup>208, 209</sup>, MHC Class I and II<sup>215, 216</sup> and 3) viruses; HIV<sup>208</sup>, SIV<sup>217</sup>, FIV<sup>218</sup> and SV40<sup>219, 220</sup>.

At the time of the discovery of NF- $\kappa$ B it was apparent that it was active constitutively in some cells and inducible in others. Furthermore, induction did not require *de novo* protein synthesis suggesting NF- $\kappa$ B remains inactive in quiescent

cells<sup>221</sup>. The presence of pre - existing NFκB and an inhibitor, IκB, was thus hypothesized<sup>219</sup>.

Early studies on NF-κB required isolation of protein from cells<sup>222</sup>, but in 1990 genes coding κB binding proteins, up - regulated on cell activation, were found<sup>223-226</sup>, coding a peptide of 105kD cleaved to a product of 50kD - NF-κB p50. The COOH terminus of p105 contains ankyrin repeats which may inhibit transcriptional up - regulation by keeping it in the cytoplasm<sup>225-227</sup>. In a lymphoma associated translocation these ankyrin repeats are deleted from p105 and NFκB is constitutively active<sup>228</sup>. Transcription of p105 message is increased after cell activation<sup>223, 227</sup> resulting in an additional level of activation<sup>182</sup>.

p50 has strong homology with the oncogene *Rel* and a *Drosophila* developmental protein, dorsal. The human oncogene *lyt 10* has homology to NFκB peptides and when translocated into immunoglobulin enhancers can drive B cell lymphomata<sup>228</sup>. The archetypal v-rel protein, from transforming strains of an avian retrovirus, binds κB DNA motifs but inhibits transcription, suggesting some Rel family members antagonise NFκB<sup>229</sup>. p50 contains a DNA binding site, nuclear localisation signal and a protein kinase A (PKA) substrate domain, is unable to up - regulate transcription on its own<sup>226</sup> and instead inhibits transcription<sup>216, 230</sup>. For transcriptional activity, p50 requires a second peptide, p65<sup>231</sup>. This peptide is also a Rel family member, associates with IκB during quiescence, probably does not bind DNA and forms (p50)<sub>2</sub>(p65)<sub>2</sub> tetramers *in vivo* conferring transcriptional activity to p50<sup>224, 225, 229</sup>. The transcription activating properties of NFκB reside in p65 and so high mobility p50 dimers are competitively inhibitory<sup>232</sup>; some T cells have increased p50 dimers and decreased NFκB reporter gene activity, representing another regulatory mechanism<sup>233</sup>.

A third inducible 86kD T cell protein, originally discovered binding to κB motifs in the HIV promoter and subsequently to the IL2Rα gene<sup>208, 234</sup>, is HIVEN86<sup>235</sup>. In other studies HIVEN86 expression appears to be constitutive<sup>236</sup>. H2TF1 is a constitutively active protein<sup>212</sup>, which preferentially binds the palindromic κB motif<sup>237</sup> in the MHC Class I enhancer<sup>220</sup>, although its role in HIV activation is unclear.

IκB consists of a group of 37-60kD proteins<sup>238</sup>, containing ankyrin repeats<sup>239</sup> and released from NFκB by detergents. IκB is synthesised after cell activation<sup>240</sup>, presumably as part of a negative feedback mechanism and is homologous to the oncogene *Bcl -3*<sup>232</sup>. The major IκB species, IκBa and IκBb, interact predominantly with NFκB p65<sup>239</sup>. IκB is phosphorylated on cell activation by PKC and PKA<sup>238</sup>, which dissociate it from p65<sup>241, 242</sup>. A cloned IκB protein, MAD-3, additionally contains a tyrosine kinase site<sup>240</sup>. Since NFκB activity is increased in CD45- T cells<sup>243</sup>, it has been proposed that CD45 phosphatase inhibits the dissociation of IκB from NFκB by keeping the former phosphorylated<sup>243</sup>.

ROIs also facilitate IκB/p65 dissociation and may explain how TNF induces NFκB binding<sup>192</sup> independent of protein kinases<sup>189</sup>. Confusingly, *in vitro* NFκB binding is reduced in oxidising conditions, presumably because the peptides are

vulnerable to oxidative conformational change<sup>244-246</sup>. Since neither free IκB nor NF-κB are found in cytoplasm, they must be present in stoichiometrical amounts. Despite the ankyrin repeats, IκB can diffuse into the nucleus, bind to p65 and dissociate NFκB from DNA; this may explain why NFκB binding is only transient after cell activation<sup>239</sup>.

The final group of proteins with κB binding activity are transcription factors containing zinc fingers. These bind the κB motifs in the HIV enhancer<sup>247, 248</sup> and can be constitutive (eg PRD-BFII) or inducible (eg PRD-BF1/MBP1/HIV-EP1/AGIE-BP)<sup>249-251</sup> by *de novo* transcription<sup>252</sup> after cell stimulation including by TNF<sup>253</sup>. These proteins contain two sets of zinc fingers, each of which can bind a κB motif<sup>251</sup>. The HTLV-1 transactivating gene product, Tax, also codes a protein which appears to have a zinc finger like region<sup>254</sup>.

Proteins encoded by viral genes also up - regulate transcription through NFκB; this requires co - infection of the same cell and is discussed more fully in chapter 7.

In T cells, NFκB can be induced by a wide variety of stimuli. In some instances, these effects are additive, for example TNF and PMA, presumably using both ROIs and PKC respectively<sup>255, 256</sup>. The use of two pathways may explain, how these types of stimuli may have synergistic effects on the up - regulation of the IL2 receptor<sup>146</sup>. The specificity of transcriptional changes following NFκB induction is probably governed by neighbouring motifs in the enhancer (see below) and by the different affinities of different κB motifs for transcription factors<sup>238</sup>. Stimuli known to activate via NFκB in T cells are shown in Table 1.2

Table 1.2 Induction of NFκB in T cells.

Stimulus	Mode of action	Ref.
Phorbol ester	PKC	221
Anti CD3	PKC	72, 257
Antigen	??	257
Anti TcR	??	257
PHA	??	221
Anti CD2	??	258
IL1	?PKA	259
IL2	??	257
TNF	ROIs	189, 255, 260-262
H <sub>2</sub> O <sub>2</sub>	ROIs	192
Ultra violet	??	263
CD45 phosphatase	?inhibits IκB dissociation	243
CD28	??	72, 74, 75
Okadaic acid	Phosphatase inhibition	264
HTLV1 tax	??	265
HIV	p105 transcriptional up - regulation.	266

HIV	p105 proteolysis	267
Herpes viruses	Multiple transcription factor induction.	268, 269

The induction of NFκB can be summarised as follows; cell surface events induce PKC, PKA, ROIs or tyrosine kinases, which release (p50)(p65) from IκB. These processes may require phosphorylation of IκB, which may be antagonised by CD45. (p50)<sub>2</sub>(p65)<sub>2</sub> tetramers form and migrate to the nucleus, bind κB sites and mediate transactivation through p65. A second regulated wave of induction is the synthesis of new p105 and its controlled processing to p50. Down regulation occurs if excess p65 or newly synthesised IκB inhibit NFκB binding.

### 1.3.4.2 Other T Cell Transcription Factors.

NFAT1 is an inducible transcription factor for IL2<sup>270, 271</sup> and for HIV<sup>272</sup>, although it was originally thought to be T cell specific. NFAT binds to specific purine rich motifs<sup>206</sup>. It is induced after signalling by PMA with calcium ionophore<sup>273</sup>, anti - TcR, PHA<sup>270</sup>, but not TNF<sup>260</sup> and requires PKC activity<sup>271</sup> and protein synthesis for its induction<sup>256, 270</sup>. Induction of NFAT and, to a much lesser extent, NFκB are blocked by immunosuppressive drugs cyclosporin A and FK506<sup>274</sup>. These bind to peptidyl isomerases involved in calcium signal transduction<sup>275</sup>, resulting in decreased expression of both IL2 and IL2Rα<sup>276</sup>. Studies using cyclosporin A suggest that synthesis of a nuclear component of NFAT follows PKC activation whilst calcium influx induces nuclear migration of a pre existing cytoplasmic moiety<sup>277</sup>.

A second protein, ILF, distributed more widely than NFAT and homologous to the drosophila regulatory protein forkhead<sup>272</sup> also binds NFAT motifs and is expressed constitutively, explaining the widespread activation of NFAT-reporter genes in transgenic mice<sup>273</sup>.

Oct1 is a ubiquitous factor which constitutively binds the IL2 enhancer<sup>270, 271</sup> but requires co-operation from other binding proteins to up - regulate transcription<sup>278</sup>.

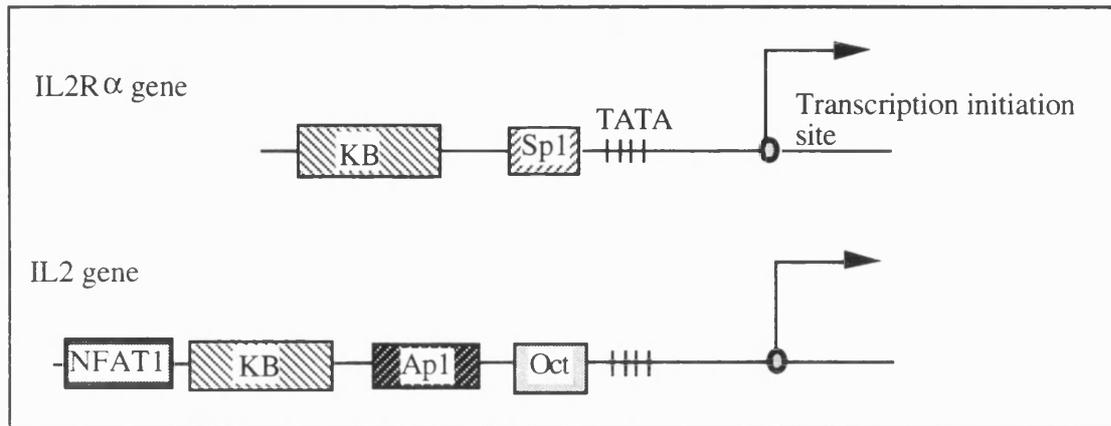
Sp - 1 is a ubiquitous zinc finger containing transcription factor<sup>279</sup>, frequently expressed constitutively, which binds onto motifs near the transcription initiation site<sup>280</sup>. Sp - 1 is required for transcription of many viral and cellular genes<sup>280</sup>, facilitating other more distally binding transcription factors<sup>268, 281</sup>. Hence Sp - 1 can compensate for decreased amounts of NFκB<sup>282</sup>.

AP - 1 is a transcription factor required for IL2<sup>206</sup>, IL6<sup>210</sup> and HIV<sup>283</sup>; AP - 1 activity is increased by ultra violet<sup>263</sup>, TNF<sup>188</sup>, and HTLV1 Tax<sup>254</sup> independently of ROIs<sup>192</sup>. AP - 1 induction is resistant to cyclosporin and requires increased synthesis of its component oncogene products jun and fos<sup>188</sup>.

The TATA box motif is situated close to the transcription initiation site and binds cellular factors TFIIA - TFIIF, promoting RNA polymerase mediated transcription. This mechanism is frequently used by viruses<sup>284,285</sup> in addition to cellular

genes. HIV has evolved a specialised TAR region which can use binding of this group of proteins<sup>279</sup>.

Figure 1.6. The IL2 And IL2R $\alpha$  Enhancers.



The IL2 and IL2R $\alpha$  genes are paradigmatic T cell activation genes. Transcription of both genes requires NF $\kappa$ B binding<sup>75</sup> which is sufficient for IL2R $\alpha$  up - regulation, whilst IL2 requires additional transcription factor binding<sup>256</sup>. Hence signals that result in induction of only one transcription factor may not stimulate T cell proliferation in all cells<sup>278</sup>.

Regulation of these genes occurs at multiple levels; depending on the type of stimulation, transcription factors are induced which rapidly migrate to the nucleus and bind enhancer motifs, possibly displacing inactive constitutively expressed proteins. In the case of IL2, the correct constellation of proteins must be in place for transcription to commence. Newly synthesised factors, for example NF $\kappa$ B provide a second wave of up - regulation. Activation is down - regulated as inhibitory factors, such as I $\kappa$ B, which may be newly synthesised, migrate to the nucleus.

## 1.4. Immunological Memory.

Hypo - responsive naive T cells are stimulated, in an appropriate manner, and subsequently increase in number and acquire a lower activation threshold. These cardinal features of immunological memory, clonal expansion and acquisition of hyper - responsiveness, acquired after primary antigen exposure, promote a stronger more rapid response on re - exposure. For example, intact humans respond to antigenic vaccine proteins by developing long-standing protection against pathogens. Such *in vivo* responses may provide the most biologically relevant means of testing immunological memory. Immunological disorders, such as that caused by HIV, impair the acquisition and maintenance of immunological memory in demonstrable ways, such as failure to respond to vaccine<sup>286-288</sup>.

*In vivo* hypo - responsive cells may receive co - stimulatory signals from APCs and other more hyper - responsive lymphocytes during the immune response (reviewed in 289). When attempts are made to identify the number of responding cells *in vitro* hypo - responsive cells may lose the help of hyper - responsive cells and be less able to

respond. Hence *in vitro* assays and limiting dilution analysis in particular, may be more tests of hyper - responsiveness than clonal expansion<sup>290, 291</sup>.

Although *in vitro* assays are thus limited, when used in investigations of immunological memory, they have permitted the assembly of the current view of T cell memory and its relationship to CD45.

### 1.4.1 CD45 And Components Of Immunological Memory.

The peripheral T lymphocyte population can be divided functionally into naive and memory cells. Naive T cells are post thymic lymphocytes which have yet to be stimulated by their specific antigen and proliferate weakly with delayed kinetics after primary antigen exposure. Memory cells have been antigenically stimulated and on re - exposure proliferate strongly and rapidly<sup>292</sup>. Secondary characteristics of memory cells are that they should traffic preferentially through tissues in addition to lymphoid organs<sup>293, 294</sup>, and that they should have a broader range of effector functions, notably cytokine secretion<sup>78, 295</sup> that persists for long periods after priming<sup>23</sup>.

Lymphocytes from neonates are naive *per se* and a progressive increase in memory T cells would be expected during adult life. CD45 isoforms bearing the A exon predominate in neonatal human TcR  $\alpha\beta$ <sup>+</sup><sup>296, 297</sup> and TcR  $\gamma\delta$ <sup>+</sup> cells<sup>298</sup> and sheep T cells<sup>293</sup>, to be replaced until equilibrium by cells expressing CD45RO. A subset of CD45RA<sup>low</sup> RO<sup>low</sup> cells from cord blood has recently been identified<sup>299</sup>, but when these are cultured *in vitro*, they also up - regulate CD45RA expression. In mice loss of CD45RA is only seen in the CD8<sup>+</sup> subset and not at all with CD4<sup>+</sup> cells; few murine CD4<sup>+</sup> cells express CD45RA to begin with<sup>291</sup>. However, lack of CD45RO monoclonal antibodies and dissimilarities of CD45RA expression make mouse data incomparable. A view emerges that the CD45RA isoform is expressed on naive T cells and CD45RO on antigen experienced cells.

These data were reinforced by the finding that upon activation with mitogen T cells switch CD45 isoform expression from RA to RO<sup>297, 300</sup>, suggesting that populations expressing these isoforms are of the same lineage but of a different maturity / excitation state. Similarly, cord blood CD45RA<sup>low</sup> RO<sup>low</sup> cells only transiently express CD45RA before going on to express high levels of CD45RO upon stimulation with mitogen<sup>299</sup>. Situations in which CD45RA<sup>+</sup> cells retain CD45RA after adequate stimulation, for example with cytokines<sup>301</sup>, appear to be unusual. When CD45RA<sup>+</sup> T cells are stimulated with phytohaemagglutinin (PHA), they not only acquire CD45RO but also the ability to help B cells in a pokeweed mitogen (PWM) system<sup>302</sup>. In contrast, some support is lent to the notion that CD45 isoform expression represents difference in lineage rather than maturity by the finding that some cells remain CD45RA<sup>+</sup> after ConA stimulation and do not acquire B cell help<sup>303</sup> and that PWM stimulation does not result in the ability of CD45RA<sup>+</sup> cells to secrete memory type cytokines<sup>304</sup>.

When cells are separated into populations expressing either CD45RA or CD45RO, only the latter respond to recall antigens in both bulk culture<sup>297, 305</sup> and limiting dilution analysis<sup>306, 307</sup>. For the reasons mentioned above, conditions in these experiments may not provide adequate co - stimulatory signals to elicit responses in the CD45RA+ cells and so only imply that this population is hypo - responsive<sup>291</sup>. A pertinent example of this is that CD45RA+ cells require co - stimulation with IL6 in order to respond to some stimuli (for example anti - CD2), a cytokine only secreted by CD45RO+ T cells<sup>308</sup>. Similarly, CD45RA+ cells are able to respond to allo - stimulation as well as their CD45RO+ counterparts<sup>300, 306, 307</sup>, possibly because the allogeneic stimulating cells are able to provide co - stimulation with adhesion molecules<sup>70</sup> or cytokines<sup>96</sup>. Only recently have responses to antigen been seen in the CD45RA+ population<sup>292, 309</sup> and then with kinetics which suggest a primary response.

Much less is known about correlates in human CD45RB expression. CD45RA+ cells all express high levels of CD45RB<sup>49, 310</sup>, whilst CD45RO+ cells can be divided into CD45RB<sup>high</sup> and CD45RB<sup>low</sup> sub - populations<sup>310</sup>. On activation the majority of CD45RA+ RB+ cells retain CD45RB<sup>310</sup> and within the CD45RO+ population, RB<sup>high</sup> proliferate and secrete  $\gamma$ IFN rather more than the CD45RB<sup>low</sup> population, in response to mitogen, suggesting RB<sup>high</sup> cells resemble T<sub>H</sub>1 cells<sup>310</sup>.

## Population Dynamics Of CD45 Expression

Stimulation of CD45RA+ cells, tested *in vitro* with mitogen, results in proliferation and switching to CD45RO expression<sup>297, 300</sup>. Activation of CD45RO+ cells also results in proliferation with maintenance of CD45RO expression. Hence, following activation there is an increase in the number of cells, meeting the first criterion for immunological memory, and a shift to CD45RO expression.

As mentioned above, the number of circulating CD45RO+ T cells increases to equilibrium during early life, consistent with growing immunological experience. Except during acute viral infections<sup>311, 312</sup>, the ratio of circulating CD45RA:RO cells in adult humans is maintained at approximately 1:1 and the size of the T cell pool is static. This is true even in the absence of the thymus<sup>313</sup>, implying the presence of peripheral homeostatic mechanisms. The net shift from CD45RA to CD45RO expression cannot cease, since adults continue to mount responses to neo - antigens, so either CD45RO+ cells revert to CD45RA expression, CD45RO+ cells die more frequently or CD45RA+ cells proliferate without switching to CD45RO+ expression, as recently described in a cytokine driven system<sup>301</sup>.

Peripheral T cells do not revert to CD45RA expression after *in vitro* stimulation, but may re - up - regulate CD45RA+ and become double positive RA+/RO+<sup>313, 314</sup>. The idea that human peripheral CD45RO+ T cells revert to CD45RA expression *in vivo* is supported, to some extent, by the finding that the number of CD45RA+ cells with DNA induced injury increases transiently after X irradiation, concomitant with a decline in the number of injured CD45RO+ cells<sup>315</sup>. These lymphocyte life span data have been

interpreted to suggest that recently activated CD45RO+ cells regain CD45RA and contribute to the apparent longevity of this population, although irradiated cells may not behave normally.

This model predicts that the CD45RA+ pool is a mix of naive and memory cells and should contain cells capable of mediating long term memory, at least to rarely encountered antigens. Although conventional limiting dilution assays show no secondary responses in CD45RA+ populations<sup>306,307</sup>, CD45RA+ cells do produce primary responses to neo - antigen, i.e. delayed responses of low amplitude<sup>292</sup>. In these CD45RA+ bulk cultures, responses were also seen to recall antigen although these too had primary response characteristics<sup>292</sup>. These CD45RA+ cells responding to recall antigen are either cells that never left the CD45RA+ pool after primary stimulation or cells which transiently resided in the CD45RO+ pool and then re - expressed CD45RA and in doing so became hypo - responsive.

Mouse *in vivo* data show memory responses apparent in the CD45RA+ pool 20 days after priming<sup>290</sup> suggesting a rapid return to this phenotype. These data do not translate well into a human model of memory since the CD45RA distribution is dissimilar in mice and humans. It has also been established that switching from CD45RC+ to CD45RC- and *vice versa* occurs *in vivo* in rats<sup>316,317</sup>, using the antibody Ox 22. CD45RC expression has been correlated with CD45RA in rats (A.N. Barclay, personal communication) and CD45RB in humans (P. Beverley, personal communication) making these data also difficult to fit into a functional memory framework. At best these data support the idea that CD45 isoform switching need not be permanent, although conclusions about functional correlates cannot be drawn.

The model that emerges is that after antigenic stimulation proliferating T cells acquire CD45RO along with co - stimulatory molecules which reduce the activation threshold. Hence the cardinal features of memory, clonal expansion and hyper - responsiveness are acquired. The fate of these cells then depends on environmental factors. Continued expression of antigen or the presence of cross reactive antigen allows these cells to remain in the hyper - responsive state, whilst reduced stimulation can lead to apoptosis.

After stimulation cells may become activated and proliferate. The resulting progeny may undergo apoptosis, be maintained in an activated state or return to a quiescent state. In some situations activation is abortive and cells become anergic; these possible outcomes and their implications for memory are discussed below

Cell death as a mechanism for regulating the T cell pool appears to be especially important in the CD8+ subset. In the course of acute Epstein Barr and Varicella Zoster virus infections apoptosis is seen in T cells expressing CD45RO<sup>312</sup>. Early HIV infection is associated with increased circulating CD8+ CD45RO+ cell numbers<sup>318</sup>, which are not sustained throughout the natural history of this infection<sup>319</sup>. Measurable proportions of these CD8+ T cells undergo apoptosis during very early HIV infection<sup>320</sup> and the number of apoptosing T cells declines as the infection enters the chronic asymptomatic stage<sup>320</sup>. The size of the CD8+ population may be restrained in this setting by the

apoptosis which has been observed to occur<sup>312, 321, 322</sup>. This tendency to apoptosis may occur especially in CD45RO+ cells expressing reduced amounts of CD45RB, markers possibly associated with advanced maturity or priming<sup>52</sup>.

The molecular basis for apoptosis occurring preferentially, at least in the activated fraction of CD45RO+ cells, may be that they express decreased amounts of bcl - 2<sup>52, 312</sup>, increased amounts of Fas<sup>52, 322</sup> and secrete diminished amounts of IL2<sup>52</sup>. Furthermore, signalling through Fas is stimulatory in fresh, resting T cells and only results in apoptosis after long term antigen exposure or in malignant T cell lines<sup>196</sup>. It should be noted that apoptosis not only prevents over expansion of the CD45RO+ pool, but may contribute to refinements in specificity<sup>323</sup> (see below).

Apoptosis may also abnormally decrease the size of the lymphocyte pool and it has been proposed that the very high expression of Fas by lymphocytes from patients with systemic lupus erythematosus may result in the observed lymphocytosis<sup>426</sup>. The presence of marked T cell apoptosis in HIV infection has been proposed as a mechanism for CD4+ lymphocyte depletion in the pathogenesis of AIDS rather than the regulation of the immune response. The fact that apoptosis affects predominantly CD8+ cells suggests that it makes only a minor contribution, if any, to CD4+ depletion. Apoptosis following activation has been proposed as a means of restraining the immune response; excessive proliferation could result in malignancy, toxic side effects or dilution of virgin cells<sup>419</sup>. Prior to the suggestion that memory T cells are short lived<sup>315</sup>, it was thought that memory cell longevity and not antigen persistence was the basis of immunological memory<sup>324</sup>. A different view<sup>325</sup> is that persistent antigen maintains memory. In addition, antigen retained on follicular DCs may be presented to B cells and subsequently to T cells<sup>326-328</sup>. This latter mechanism only supports memory T cells<sup>329</sup>, possibly because they are capable of being stimulated by the low level of B7 on B cells<sup>8</sup>. Limiting quantities of persisting antigen may then select clones of T cells with higher affinity receptors, thus effectively promoting a kind of T cell "affinity maturation"<sup>325</sup>, which has been demonstrated in CD8+ cells<sup>419</sup>. Cross reactive antigen can continue stimulating CD45RO T cells, because of up - regulation of co - stimulatory molecules. Responses may then spread to antigens to which the individual has not been exposed or auto - antigens<sup>294, 325</sup>. These two mechanisms rely on interleukin secretion to prevent apoptosis<sup>312, 323</sup>.

Other T cells appear to revert to a more hypo - responsive state and re - up - regulate CD45RA, without losing CD45RO and avoiding apoptosis; this may be promoted by the presence of fibroblasts, which allow return to the quiescent state without cell death (Diana Wallace, personal communication)<sup>312, 323</sup>. How frequently this occurs is unclear, but if these populations are significant then antigen experienced cells should be detectable in the CD45RA+ population. Cell cycling is most common in CD45RO+ cells<sup>293</sup> and the half life of CD45RO+ cells is short<sup>315</sup>, implying that although activated cells retain the RO isoform whilst cycling, while the majority return to the CD45RA state on becoming quiescent.

Another possible outcome of T cell activation is that cells become anergic, which may occur when CD45RO+ cells are presented with antigen by abnormal APCs<sup>330</sup>. The APC molecule responsible for preventing anergy induction may be the CD28 ligand B7<sup>77</sup>, although this is also important in providing co - stimulation to CD45RA+ cells. Since anergy induction is preventable with cyclosporin<sup>331</sup>, it may be that it represents a state of partial but inadequate activation.

Hence following acquisition of the CD45RO+/memory phenotype, a number of possible outcomes may maintain different types of long term memory, possibly in both the CD45RO+ and, in a modified form, CD45RA+ pools.

### 1.4.3 CD45 And Hyper - Responsiveness.

The second cardinal feature of immunological memory is hyper - responsiveness, measured by studying activation in isolated populations after mitogen stimulation. Hyper - responsiveness can be explained by differences in the T cell signal transduction mechanism itself or by co - stimulatory requirements of the populations.

No quantitative data are available for the expression of TcR, CD4 and CD8 in naive and memory populations (see chapter 3) and most attention has focused on CD45 itself (see sections 1.2.3 and 1.2.6). In the mouse CD45 associates with the signalling complex only on activated T cells<sup>39</sup>, whilst in humans CD45 isoforms associate differently with co - stimulatory molecules<sup>40</sup>. Although CD45RO+ cells have higher basal levels of DAG and PKC, attributed to recent *in vivo* activation in these experiments<sup>136</sup>, and CD45 may regulate cluster formation involving LFA- 1<sup>55,56,64</sup>, there is no data to support the idea that CD45 isoforms directly regulate T cell activation signals differentially.

Hyper - responsiveness of CD45RO+ cells is also conferred by different needs for co - stimulatory signals, either surface molecules or cytokines provided by APCs or other lymphocytes. CD45RA+ T cells can proliferate at least as well as their CD45RO+ counterparts in response to PHA, PWM, super - antigen and allogeneic stimulation<sup>69, 295, 332</sup>. Other polyclonal stimulants preferentially activate the CD45RO sub - population, but only under conditions of minimal co - stimulation. For example, the response to soluble anti - CD3 is generally greater in the CD45RO+ CD4+ sub - population<sup>69, 70, 333</sup>. When the stimulatory signal is boosted by increasing the concentration of the CD3 antibody<sup>69</sup> or by immobilising it<sup>295</sup>, CD45RA and CD45RO populations proliferate comparably. The response of the CD45RA sub - population can also be augmented by adding co - stimulatory signals such as anti - CD28<sup>70</sup>, increasing ICAM - 1 on accessory cells<sup>334</sup>, IL2<sup>333</sup>, IL1 or TNF<sup>96</sup>. The CD45RA+ CD4+ response to CD2 antibodies is also increased to the level of CD45RO+ CD4+ cells when IL-2, IL-6<sup>308</sup> or IL4<sup>335</sup> are added. IL1 augments the CD45RA+ T cell response to neo - antigen<sup>309</sup>. Finally, CD45RO+ T cells can respond to IL2 and TNF without the need for TcR/CD3 mediated stimulation, whilst CD45RA+ cells additionally require IL6<sup>301</sup>.

These data have been interpreted as evidence of "hypo - responsiveness" of the CD45RA sub - population within the naive/memory conceptual framework<sup>70</sup>.

In the soluble anti - CD3 stimulation model, TK signals are the same in both CD45RA+ and RO+ cells, although proliferation is greater in the former. This has been interpreted as showing CD3 is coupled equally well to the signalling mechanism in both populations<sup>135</sup>, but that second signals required for commitment to proliferation occur at a lower threshold in CD45RO+ cells<sup>136</sup>. In part these differences also reflect the low level of co - stimulatory molecules on CD45RA+ cells, for example CD2, LFA - 1, and VLA 4, which are up - regulated only after transition to CD45RO expression<sup>86,97</sup>,

The need for co - stimulation restricts the type of cells which can present antigen to the CD45RA+ population<sup>336</sup>, so that CD45RA+ cells respond most effectively to DCs<sup>10, 13, 76</sup>. The efficiency of DCs at presenting to CD45RA+ cells is largely due to high expression of B7<sup>7, 8, 76</sup> and MHC Class II<sup>423</sup>. Although DCs do not secrete IL1<sup>424</sup> and can stimulate CD45RA+ cells in the absence of this cytokine<sup>337</sup>, IL1 can augment fixed DC co - stimulatory ability<sup>338</sup>. Activated monocytes secrete ample amounts of TNF and IL1<sup>96, 240</sup> and express intermediate levels of B7, but are said not to induce primary responses efficiently<sup>10</sup>. Lack of IL1 and inadequate B7 expression on resting B cells may explain why these cells cannot present to naive T cells<sup>329</sup>. IL4<sup>27, 69, 339</sup> and IL6<sup>27, 295</sup> among T cells are secreted exclusively by those expressing CD45RO, which co - stimulate CD45RA+ cells in a shared milieu. This may contribute to the lack of recall responses in purified CD45RA+ cells.

Antigen can be presented to CD45RO+ T cells by B cells<sup>329</sup>, monocytes<sup>76</sup> and CD8+ T cells in addition to the professional APCs that present to CD45RA+ cells. Antigen presentation by B cells gives CD45RO+ T cells access to long term antigen stores on follicular DCs (reviewed in 326-328). CD45RO+ cells are not independent of the need for co - stimulation; they require adequate IL2 secretion, largely from CD45RA+ T cells<sup>27, 69, 339</sup>, to proliferate optimally<sup>300, 306</sup> and prevent apoptosis<sup>312, 323</sup> and B7 expression on APCs<sup>77</sup> to prevent anergy induction after partial activation.

#### 1.4.4 CD45 And Patterns Of Cytokine Secretion

The differences in cytokine secretion in human CD45RA+ and RO+ T cells have been alluded to above and are listed in Table 1.3. It must be noted that the data summarised in Table 1.3 are all from activated T cells, so cytokine secretion patterns may reflect both the mitogen and APC type used. It can be seen that CD45RA+ cells secrete largely IL2 alone whilst both T<sub>H</sub>1 and T<sub>H</sub>2 cytokines are secreted by CD45RO+ cells.

To an extent, T<sub>H</sub>1/T<sub>H</sub>2 cytokine secretion patterns segregate with expression of CD45 isoforms in rodents<sup>49, 341</sup>. Cytokine secretion patterns are much less clear in human CD4+ cells<sup>27, 28</sup>, although, within human CD45RO+ cells,  $\gamma$ IFN secretion appears to be increased in CD45RB<sup>high</sup> rather than RB<sup>low</sup> cells<sup>52, 310</sup>. Although these may be maturational changes, it remains possible that high CD45RB expression is a marker of T<sub>H</sub>1 phenotype in man.

**Table 1.3. Cytokine Secretion By CD45RA+ And CD45RO+ CD4+ Lymphocytes.**

Cytokine	CD45RA+	CD45RO+	Reference
IL2	++++	++	27, 28, 69, 304, 339
IL4	-	++++	27, 28, 69, 304, 339
IL6	-	++++	27, 295
$\gamma$ IFN	+	++++	27, 28, 69, 297, 304, 332
TNF	+	+++	340

The data discussed in section 1.4.3. emphasise how two types of co - stimulatory signals are required in all stages of peripheral T cell maturation. DCs can present to cells which eventually produce both T<sub>H</sub>1 and T<sub>H</sub>2 progeny<sup>342</sup>. Since T<sub>H</sub>1 and T<sub>H</sub>2 clones appear in the CD45RO+ population<sup>27</sup> specialisation must occur within this compartment. The evolution of either T<sub>H</sub>1 or T<sub>H</sub>2 clones may rely on the specialised co - stimulation by macrophages or B cells, respectively, to achieve further degrees of differentiation after the expression of CD45RO.

### 1.4.5. CD45, Adhesion And T Cell Migration Pathways.

The adhesion molecules discussed in section 1.2.5. are involved in the regulation of T cell migration and the formation of cellular clusters, most importantly with APCs. The function of these molecules differs in CD45RA+ and CD45RO+ T cells and has a considerable impact on immunological memory.

Homotypic and heterotypic adhesion are mediated by molecules which can be regulated quantitatively and qualitatively. CD45RO+ cells express up - regulated LFA - 1, LFA - 3, CD2, ICAM - 1 and VLA4<sup>86, 297</sup>. LFA - 1 affinity is increased after T cell activation and decreased by CD4 binding, processes which are regulated differently in cells expressing CD45RA or CD45RO<sup>56</sup>. This may be a consequence of different CD45 isoform expressed since CD45 isoforms co - cap with different adhesion molecules (see section 1.2.3), some CD45 antibodies augment LFA 1 mediated cell clustering<sup>91</sup> and others inhibit clustering involving other adhesion molecules<sup>55, 128</sup>. Thus, CD45 isoform expression may have an impact on processes involving cell clustering, such as HIV syncytium formation.

Compartmentalisation appears to not only affect cytokine secretion in populations defined by CD45 isoform expression, but also migration pathways and physical location of T cells. Hence naive T cells circulate from blood, through high endothelial venules (HEVs) in uninflamed lymph nodes and into efferent lymphatics<sup>293, 294</sup>. Memory cells migrate from blood into tissues and then into afferent lymph; few CD45RO+ cells are found in efferent lymph, except in active lymph nodes.

Lymphocyte exodus takes place in a step wise manner. Low affinity selectin interactions initiate a slow rolling movement which allows lymphocytes to bind chemokines, soluble factors released by endothelium<sup>89</sup>. These up - regulate binding between integrins (LFA - 1, VLA4, LPAM) and their endothelial ligands. This inside out signalling may confer temporarily increased affinity, particularly in CD45RO+ cells<sup>56</sup>. CD45RO+ cells also have enhanced ability to open intercellular gaps in the endothelium, possibly via cytokine secretion<sup>159</sup>. Selectin binding, sensitivity to chemokines and integrin expression provide a means by which a hierarchical code may direct T cells to specific tissues<sup>90</sup>.

CD45RA+ lymphocytes are homogeneous in their expression of selectins<sup>343</sup>; they express high levels of L selectin which enhances migration through lymph node HEV. Cutaneous lymphoid associated antigen (CLA) is expressed on a sub - population of CD45RO+ cells, which migrate preferentially into skin. CD45RO+ T cells have a heightened response to chemokines secreted by T cells and monocytes respectively (reviewed in Springer<sup>90</sup>).

CD45RO+ cell adhesion to endothelium is enhanced overall, by increased expression of LFA - 1 and VLA4<sup>159</sup> whilst VLA6 may promote migration through basement membranes<sup>95</sup>. Of the other integrins, CD45RO+ cells express heterogeneous levels and CD45RA+ cells high levels of LPAM - 1<sup>343</sup>, promoting migration through Peyer's patch HEV. Blood CD45RO+ are heterogeneous in their expression of homing receptors, consisting largely of two groups expressing either HML - 1 integrin or CLA and L selectin. The former group home to the skin, where they are significantly enhanced in number, whilst the latter migrate to mucosa of gut and respiratory tract<sup>343</sup>.

**Table 1.4. Homing Of CD45RA+ And CD45RO+ Lymphocytes.**

	Selectin/Ligand	Chemokine Sensitivity	Integrin	Target Organ
CD45RA+	L selectin+ CLA-	+	LPAM	HEV in lymphoid tissue
CD45RO+	L selectin- CLA-	+++	LFA - 1, VLA4 LPAM, HML	Mucosa
CD45RO+	L selectin+ CLA+	+++	LFA - 1, VLA4	Skin

Hence CD45RA+ cells migrate to lymphoid tissues where they can be presented to by the professional APCs they require for co - stimulation, whilst CD45RO+ cells migrate to sites where secondary responses are required.

An interesting paradox is that in the thymus sessile CD3- CD4- CD8-, triple negative CD45RA+ cells express more adhesion molecules than intra - thymically

mobile CD45RO+ cells. This has led to the suggestion that CD45 itself regulates whether adhesion molecules facilitate adhesion or migration<sup>344</sup>. In heterotypic adhesion, CD45RO+ cells cluster more readily, but only transiently, suggesting a negative signal disrupts the clustering advantage these cells have.

### 1.4.6 CD45 And Immunological Memory; Conclusions.

Disease processes affect the proportion of CD45RA+ and RO+ cells in blood and tissue; in auto - immune disorders there is an increase in CD45RO+ cells in affected tissue (rheumatoid arthritis, myaesthesia gravis and multiple sclerosis) and in primary biliary cirrhosis an increase in circulating CD4+ CD45RO+ cells<sup>425</sup>. Haemophagocytic lymphohistiocytosis is unique in that affected individuals and their families have decreased intensity of CD45RO expression<sup>247</sup>. Asthma is characterised by an increase in CD45RO+ cells in the bronchial lumen<sup>420, 343</sup>. Infectious processes also increase the proportion of circulating CD45RO+ cells; in moderate appendicitis there is an increase in CD45RO+ T cells in blood; the number of blood CD45RO+ T cells decreases in very severe appendicitis, concomitant with migration into tissues (P. Beverley, personal communication). In viral infections, at least, this increase in CD45RO expression affects mainly the CD8+ compartment.

In conclusion, CD45RO+ have the characteristics of memory cells:

- Clonal expansion ensures there are an increased number of primed cells to respond to secondary stimulation.
- Reduced need for co - stimulation enables CD45RO+ cells to respond to a broader range of APCs.
- A broader range of cytokine secretion allows CD45RO+ cells to exert effector mechanisms.
- In the primary response antigen must diffuse or be carried to lymph node prior to presentation to many CD45RA+ cells. The secondary response is speeded up by CD45RO+ cells *in situ* in vulnerable tissues.

### 1.5.1 Changes In Lymphocyte Populations In HIV Infection.

Numerous studies have attempted to define cell populations affected by HIV with the aim of clarifying pathogenic mechanisms and obtaining prognostic markers. These studies have analysed which populations of cells become functionally abnormal, are depleted and can be infected *in vivo* and *in vitro*. These descriptive studies on sub - populations of CD4+ T cells have provided data essential to the understanding of HIV pathogenesis and the basis to this thesis.

### 1.5.2. Abnormalities Of T Cell Function In HIV Infection.

*In vitro* studies show a continuum of what appear as T cell dysfunction, of either proliferation or cytokine secretion, although, these abnormalities could also represent abnormalities in APCs<sup>19, 345</sup>.

In very early HIV infection (after any sero - conversion illness) no abnormalities are detectable, but there is then a sequential loss, first of recall antigen response, followed by allo-responsiveness and finally response to lectin mitogens<sup>346-348</sup>. These defects are not related to total numbers of CD4 T cells, although loss of recall antigen response in asymptomatic individuals is associated with an increased risk of rapid progression to AIDS<sup>349</sup>.

Asymptomatically infected individuals also have diminished responses to anti-CD3<sup>350-352</sup> and anti-CD2 antibodies, defects which become more severe in patients with symptomatic infection and have prognostic significance in the natural history<sup>353</sup> and treatment<sup>354</sup> of HIV infection. At all stages of infection the defect can be abrogated by adding anti-CD28 or IL2<sup>352</sup>; this need for co - stimulation is similar to that seen in CD45RA+ cells. When peripheral blood lymphocytes from HIV infected patients are enriched for CD45RO cells, the responses to anti-CD3 and anti-CD2 are improved<sup>352, 355</sup>. These studies were performed in APC - independent systems and the defect was ascribed to selective loss of CD45RO T cells.

### 1.5.3 HIV And Cytokine Secretion Patterns.

HIV infection is characterised by a loss of ability to mount a successful immune response to intracellular organisms and by polyclonal B cell activation, features which have suggested a shift in regulation favouring T<sub>H</sub>2 over T<sub>H</sub>1 clones. Additional features include increased IgE levels<sup>356</sup>, an increased incidence of atopic manifestations, which may respond to  $\gamma$ IFN<sup>357</sup> and of reactive arthritides, mediated by CD4+ T cells secreting T<sub>H</sub>2 cytokines (Katja Simon, in press, *Proc. Nat. Acad. Sci.* 1995).

IL2 secretion by HIV infected T cells is defective<sup>348, 352, 358-361</sup> (although studies have also shown raised IL2 levels in *in vivo* samples<sup>362</sup>) and, in some models, impaired proliferative responses are restored by adding IL2<sup>351</sup>.  $\gamma$ IFN secretion has been found to be increased, but is derived largely from CD8+ T cells<sup>363</sup>. Fresh purified CD4+ T cells, on the other hand, contain minimal mRNA for either T<sub>H</sub>1 or T<sub>H</sub>2 cytokines, even though these cells may show features of *in vivo* activation<sup>363</sup>. Similarly, when fresh CD4+ cells are cloned with either mitogen or antigens usually eliciting a T<sub>H</sub>1 response, the emergent cytokine pattern is TH0, with secretion of both IL4 and  $\gamma$ IFN<sup>364</sup>. However, culture of CD4+ cells from tissue<sup>364</sup>, by long term cloning of PBLs<sup>365</sup> or fresh PBLs from HIV infected individuals who have lost recall response but have normal CD4+ lymphocyte numbers<sup>366</sup> show a tendency to produce T<sub>H</sub>2 type cytokine patterns. Such changes have only rarely been observed in bulk cultures<sup>367, 368</sup> and the concept emerges that although there is no distinct switch from a T<sub>H</sub>1 to T<sub>H</sub>2 cytokine secretion pattern, (residual) memory T cells tend to secrete T<sub>H</sub>2 or T<sub>H</sub>0 cytokines<sup>369</sup>.

These data are not easy to resolve with the finding that HIV preferentially replicates in T<sub>H</sub>2 cells<sup>364</sup> which should lead to premature destruction and/or dysfunction of this population (see below). The paradox may be resolved if replication is cytopathic for T<sub>H</sub>1 cells but not for T<sub>H</sub>2 cells.

T<sub>H</sub>1 cytokines have a major role in the successful immune response to viruses. HIV exposed individuals have been identified with transient IL2 responses to viral peptides but no evidence of infection, suggesting that a successful T<sub>H</sub>1 response can clear HIV in some individuals<sup>370</sup>. An argument has been constructed that HIV induces a switch towards a population of cells which secrete cytokines that are ineffective in clearing infection and are preferential targets for replication.

A number of mechanisms have been proposed to explain this putative switch in cytokine pattern. For example, T<sub>H</sub>2 cells can proliferate in response to calcium influx alone, whilst T<sub>H</sub>1 cells additionally require signalling through IP metabolism<sup>371</sup> which is known to be defective in HIV infection<sup>372, 373</sup>.

Alternatively, the primary defect may lie in APCs. Defective co - stimulatory molecule expression, particularly of B7, leads to anergy of T<sub>H</sub>1 cells<sup>345</sup> and such a change could explain why DC from infected individuals can support T<sub>H</sub>2, but not T<sub>H</sub>1, allogeneic responses<sup>374</sup>. The increased secretion of prostaglandin E<sub>2</sub> from HIV infected monocytes<sup>375, 376</sup> may favour the growth of T<sub>H</sub>2 clones<sup>377</sup>.

If dysregulation of cytokine secretion in AIDS pathogenesis is important, therapies which are known to be effective should restore T<sub>H</sub>1 cytokine secretion patterns. AZT does indeed partially restore  $\gamma$ IFN secretion in response to mitogen<sup>378</sup> and DTH responses<sup>379, 380</sup>.

The effect of HIV on cytokines is discussed more fully in chapter 7.

#### 1.5.4 CD45RO Cells Are Lost Early And Preferentially Infected In HIV Infection.

Selective depletion of sub - populations of CD4 T cell during the natural history of HIV infection has been extensively investigated. Cross sectional studies have established that the CD45RA and CD45RO sub - populations of CD4 T cells are lost at different stages. These studies are confounded by the use of CD29 reactivity as a substitute for CD45RO, because of the scarcity of antibodies against the latter<sup>297</sup>, by not always using double staining to distinguish between CD4+ and CD8+ cells, although changes in CD45 isoform expression affect these populations in different ways and not adhering to recognised clinical classifications. For example, constitutional symptoms were not recognised, in early studies, as implying progressive HIV infection.

Individuals with early asymptomatic HIV infection display only a selective depletion of the CD4+ CD29+/CD45RO+ sub - population<sup>381-385</sup>. Subsequently, patients with symptomatic HIV infection show non-selective depletion of both CD4+ CD45RA+ and CD4+ CD29+ sub - populations<sup>349, 385-387</sup>. The onset of the non - selective loss of CD4+ cells occurs along with markers suggesting disease progression;

or example, asymptomatic patients with total CD4 counts greater than 400/mm<sup>3</sup> have a selective depletion of CD4+ CD29+ cells only, whilst in patients with less than 400/mm<sup>3</sup> cells both sub - populations are depleted<sup>387</sup>. Similarly, asymptomatic individuals without detectable HIV antigenaemia have decreased numbers of CD4+ CD29+ cells, whilst the onset of antigenaemia is associated with loss of both sub - populations<sup>388</sup>. CD26 is another T cell marker associated with immunological memory; expression of CD26 is diminished from early in HIV infection<sup>389, 390</sup>.

The early loss of CD4+ CD45RO+ lymphocytes also occurs in lymph nodes. In patients with persistent generalized lymphadenopathy, who are otherwise well, there is also a selective loss of the CD4+ CD29+ phenotype, accompanied by normal populations of CD4+ CD45RA+ cells in lymph node cell suspensions<sup>391</sup>.

These changes also occur in the SIV/macaque model; a selective depletion of CD4+ CD29+ lymphocytes was seen within 2 weeks of SIV inoculation, as part of a vaccine study<sup>392</sup>. The reduction in CD45RO+ cells is predictive of disease progression and does not occur if the virus is non - pathogenic for the particular host<sup>392, 393</sup>.

The early loss of CD45RO+ CD4+ cells has been refuted by some workers<sup>394</sup>. For example, the loss of recall responses in asymptomatic HIV infected patients did not correlate with the percentage of CD45RO+ cells in the study; but when absolute lymphocyte sub - populations are used, there is a good correlation with recall function<sup>394</sup>.

A number of studies also report changes in activation marker expression on CD4+ T cells during HIV infection; IL2R $\alpha$  has been reported as being decreased<sup>350, 389, 395, 396</sup> or increased<sup>397, 398</sup> following HIV infection; MHC Class II expression may also be increased<sup>397, 398</sup> or decreased<sup>387</sup>. In asymptomatic patients there is a relative increase in the number of CD4 T cells expressing both CD45RA and CD29<sup>399</sup> reminiscent of recently activated cells prior to the up - regulation of CD45RO<sup>297</sup>. One unifying explanation is that these abnormalities represent a defect in activation during which activation markers are up - regulated, but the process is aborted or terminates in death<sup>321</sup>. However, <sup>some</sup> CD45RA+ CD4+ T cells from HIV infected patients are able to switch to CD45RO expression after mitogen stimulation *in vitro*<sup>400</sup>.

These changes in activation markers affect CD8+ as well as CD4+ T cells. Although not infected by HIV, CD8+ T cells undergo phenotypic changes in the course of HIV infection. In early HIV infection the numbers of CD8+ CD38+ CD45RO+ cells are increased in adults<sup>401, 402</sup> and children<sup>318</sup>. This increase in the size of the CD8+ CD45RO+ population has been attributed to clones of CD8 cells responding to HIV<sup>1</sup> including the HIV polypeptide gag<sup>319</sup>. However CD8+ CD45RO+ numbers do not reflect CTL function<sup>318, 319</sup> and the response to gag declines towards the late stages of HIV infection without a corresponding decline in CD8+ CD45RO+ numbers<sup>319</sup>. CD38+ CD8+ cells decline in number during treatment with zidovudine<sup>403</sup>.

### 1.5.5 HIV Infection In Sub - Populations Of T Cells.

HIV infects a small number of circulating CD4+ cells *in vivo*. Immunofluorescence and *in situ* hybridisation studies have estimated active viral infection, with expression of HIV protein and RNA, respectively, to occur in between 1/10,000 and 1/100,000 circulating T cells<sup>404</sup>. Latent viral DNA is present in between 1/100 and 1/1,000 circulating T cells. The titre of HIV DNA containing cells appears to be higher during primary infection, prior to the development of effective immunity<sup>405</sup>, and to rise again after the onset of symptomatic HIV infection<sup>404</sup>. Lymph nodes, however, appear to be a site of more florid infection, replication and cell loss<sup>406-409</sup>.

HIV is capable of *in vitro* infection of T cells at many stages in maturation, from early in thymic development<sup>410</sup> to differentiated T cell clones<sup>411</sup>, so the paucity of infected cells *in vivo* is puzzling.

*In vitro* HIV preferentially replicates in T<sub>H</sub>2 cells<sup>364</sup>; this appears to be because  $\gamma$ IFN secreted by T<sub>H</sub>2 clones inhibits replication - both populations are equally well infected containing similar amounts of HIV DNA. It is not yet clear whether this occurs *in vivo*.

Purified CD45RO+ CD4+ cells infected *in vitro* without activation contain more HIV DNA than CD45RA+ cells<sup>412</sup>. Similarly, CD45RO+ CD4+ cells from asymptomatic HIV infected patients contain 4 to greater than 10 fold more HIV DNA than CD45RA+ cells. Since CD4+ cells usually carry only one HIV DNA copy per cell<sup>404, 413</sup>, the difference in the amount of HIV carried reflects the number of cells infected. This study did not attempt to distinguish between CD45RO+ cells recently activated *in vivo* and those remaining quiescent; in fact the positive selection procedure may have activated some cells *in vitro*<sup>68</sup>. These issues are addressed in chapter 6. Interestingly, preferential infection of CD45RO+ cells also appears to occur in lymph nodes<sup>407, 414</sup>.

In pig tailed macaques asymptotically infected with SIV, virus is carried<sup>415</sup> and replicates<sup>416</sup> most frequently in CD44+ T cells; this marker is up - regulated, at least in other species, on memory/activated T cells<sup>293, 294</sup>.

The enhanced HIV replication in *ex vivo* CD45RO+ cells partly reflects the higher carriage of provirus in these cells<sup>417</sup> although a "TNF autocrine loop" has been proposed as a special mechanism to maintain replication in CD45RO+ cells<sup>340</sup>. This is re - assessed in chapter 7.

In summary, these surface marker and functional studies show that in early, asymptomatic HIV infection CD29+ CD45RO+ CD4+ lymphocytes only are lost, along with responses to recall antigens, anti - CD3 and anti - CD2 antibodies. Around the time of onset of HIV antigenaemia and declining total CD4 T cells numbers, and certainly by the time individuals become symptomatic, CD45RA+ CD4+ T cells are also lost and patients then lose the ability to respond to alloantigen and to lectin-mitogens. These phenotypic and functional studies, which suggest an early loss of cells mediating T cell memory, have been integrated into a model where "memory T cells" are preferentially infected and destroyed by HIV. This has been supported by data suggesting these cells are more readily infected *in vivo* and *in vitro*.

Proposed mechanisms for such an early loss of CD45RO+ memory CD4+ cell numbers and function have included;

- the requirements for cells to be activated or cycling for HIV binding, fusion, reverse transcription or integration<sup>418</sup>.
- enhanced transcription of HIV in CD45RO+ cells by the "TNF autocrine loop"<sup>340</sup>.
- enhanced apoptosis in this population<sup>385</sup>.
- requirement of this population for specialised APCs<sup>19, 385</sup>

In this project, some of these putative mechanisms have been investigated. Chapters 3 to 7 describe experiments which explore stages of the life cycle of HIV which may operate more or less effectively in cells expressing the RA or RO isoforms of CD45. The ultimate aim of this project was to identify features of T cell populations which enhance or are more permissive to HIV infection and may be amenable to therapeutic intervention.

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# Chapter 2. Materials And Methods

## 2.1 Preparation Of Peripheral Blood Mononuclear Cells.

Blood was separated and experiments initiated within 24 hours of collection to ensure minimal non - specific activation *in vitro*.

Buffy coats were obtained, anticoagulated with citrate, from The National Blood Transfusion Service, Colindale London or Rote Kreuz Blutspendendienst, Steglitz, Berlin. These had been screened for the presence of HIV and Hepatitis B and were found to be negative. Smaller blood samples were obtained from uninfected laboratory colleagues at low risk of HIV infection. For studies on the binding of HIV envelope glycoprotein gp120 these were defibrinated with glass beads to prevent coagulation, since heparin can inhibit binding of gp120 to CD4<sup>1</sup>.

Blood was diluted 1:3 with RPMI 1640 Medium (RPMI) and layered on top of Ficoll Hypaque (Pharmacia) prior to centrifugation at 400g for 25 minutes at 20°C. Interface cells were aspirated, washed once at 1300rpm and twice at 1000rpm to remove residual Ficoll and platelets.

Resultant peripheral blood mononuclear cells (PBMs) were re - suspended in Lymphocyte Culture Medium (LCM; RPMI, 10% Foetal Calf Serum (FCS), 2mM glutamine, 100 U/ml penicillin and 100µg/ml streptomycin) at 10<sup>7</sup>/ml and placed in Nunc 660ml Tissue Culture Flasks. Cells were incubated for 40 minutes at 37°C with 5% CO<sub>2</sub>. The non adherent cells were gently aspirated and incubated again overnight as before. After overnight culture the non adherent cells were used for cell separation, as below. The adherent cells from both these steps were pooled for use as antigen presenting cells.

In experiments studying NFκB expression, where minimal activation of macrophages by lipopolysaccharide (LPS) was required. Human serum rather than FCS was used and media were passed through a polymixin B column<sup>2</sup> (Sigma), which reduced LPS concentrations from 10U/ml to less than 0.1U/ml as detected by a Limulus Amoebocyte Lysate assay (E-Toxate, Sigma).

## 2.2 Cell Separation Into CD4+ CD45RA+ DR- And CD4+ CD45RO+ DR- Subsets And Sub - population Nomenclature.

Negative selection procedures were used throughout since monoclonal antibodies (mAbs) against surface antigens can cause abnormal cell signalling<sup>3</sup> or prevent binding of HIV<sup>4</sup>. Non - adherent PBMs were incubated in saturating volumes of UCHT4 (anti - CD8), OKM1 (anti - CD11b), BU12 (anti - CD19), L243 (anti - DR) and UCHM1 (anti - CD14) (see appendix II) in 50ml tubes (Falcon) for 30 minutes at 4°C. The cells were then washed in chilled RPMI 3 times and re suspended in 1ml of RPMI

in 15ml tubes (Falcon). Magnetic beads coated in sheep anti - mouse antibody (MT450 Dynabeads, Dynal) previously washed in RPMI were added at a ratio of 2 beads to every cell. This mixture was placed at 4°C and gently agitated every 5 minutes during a 20 minute incubation.

The presence of bead / cell rosettes was confirmed and the mixture diluted to 10ml with cold RPMI. This was then placed in a magnetic particle separator (Dynal) for 5 minutes, and the non rosetted cells aspirated. This procedure was repeated a total of three times.

The resultant CD4+ T cell enriched population was divided into two and the cells pelleted. Saturating quantities of either SN130 (anti - CD45RA) or UCHL1 (anti - CD45RO) were added. After a 30 minute incubation the cells were washed, incubated and separated as above, this time using Dynabeads at a ratio of 1:1. To avoid confusion populations are referred to as CD45RA+ (i.e. CD8- CD14- CD19- CD11b- MHC Class II-, CD45RO-) or CD45RO+ (i.e. CD8- CD14- CD19- CD11b- MHC Class II-, CD45RA-). Since expression of CD45RA and CD45RO is not absolutely different on these populations, it is in fact incorrect to refer to cells as “positive” or “negative”; however, for simplicity the terms “+” and “-” replace “high” and “low” in the following text. The purities of resultant populations were routinely checked with fluorescein - isothiocyanate (FITC) conjugated or avidin - phycoerythrin (Av PE New England Biolabs ) labelled antibodies; goat anti - mouse fluorescein isothiocyanate (GAM FITC Sigma) was not used, to avoid confusion generated by residual antibodies on cells. The purities of these populations are shown below.

**Table 1.1. Purities Of Separated Populations.**

	CD45RA+ population	CD45RO+ population
CD4+	>90%	>95%
CD45RA+	>90%	<10%
CD45RO+	<20%	>90%

In *in vitro* infection experiments, the separation procedure was modified so that CD45RO+ cells were separated into those which were MHC Class II positive and negative by omitting L243 (anti - DR) from the first cocktail of antibodies. Enriched CD4+ cells were treated, in the second step, with UCHL1 and L243, SN130 and L243 or SN130 alone; the resulting populations were designated CD45RA+, CD45RO+ ClassII- or Class II+. The characteristics of the resultant populations are shown in chapter 6.

## 2.3 Cytofluorimetry.

### 2.3.1. Immunofluorescence.

All immunofluorescence (IF) staining procedures were carried out at 4°C in staining medium (PBS-A with 1% bovine serum albumin containing 0.1% sodium azide). For indirect fluorescence, cells were stained with a saturating concentration of mAb in the presence of staining medium. In some cases the antibodies were biotinylated (see below). After 30 minutes at 4°C, cells were washed three times and incubated with either GAM FITC or Av PE. After 30 minutes, cells were washed three times. For direct IF, cells were stained in one step with FITC conjugated mouse antibody. In either case, cells were fixed in 1% formaldehyde in staining medium.

Analysis was performed on a FACScan (Becton Dickinson) with a live gate set on forward scatter. For two colour staining, singly stained populations were analysed first, to set the compensation. Between 5 - 10,000 cells were acquired per sample and data were analysed on Lysis II software.

### 2.3.2. Measurement Of Intracellular Calcium.

In these experiments, a FACStar equipped with two argon lasers, one tuned to ultra - violet, was used to analyse fluorescence at three wavelengths. Rather than physically separate populations, cells were stained with a cocktail of antibodies to exclude contaminating monocytes, NK cells, B cells, CD8+ cells and cells expressing either CD45RA or CD45RO (to leave either CD4+ CD45RA+ or CD4+ CD45RO+ cells unstained). The advantage of studying only the unstained population was that there was no interference in the emission spectra of Indo 1<sup>5-7</sup>.

Fifty micrograms of Indo 1 (Molecular Probes) was initially dissolved in 180µl DMSO with 20µl 1.25% Plurionic acid (Sigma) and then added at 1:100 to the cell suspension at 2.5x10<sup>6</sup> cells/ml. The cells were incubated at 37°C for 20 min and then washed three times. Reciprocal IF staining was performed by incubating the cells with saturating concentrations of UCHT4, OKM1, BU12, L243 and UCHM1 along with either UCHL1 or SN130. After a 30 minute incubation, and three washes, reciprocal staining was completed with FITC conjugated goat anti mouse antibody. Prior to analysis, cells were briefly brought up to 37°C and then mixed with either UCHT1 (anti - CD3), gp120 or medium alone.

Analysis was performed on a FACStar (Becton Dickinson) after excitation at 360nm. Green emitting cells (from the antibody cocktail/FITC) were gated out and data from emissions in violet (400nm) and blue (485nm), representing calcium bound and free Indo 1, respectively, were collected. The ratio of the intensity of these two emissions is proportional to intracellular calcium. Software presented data either as the mean fluorescence ratio or the percentage of cells with an increasing ratio, in real time.

### 2.3.3. Measurement Of Intracellular Glutathione.

Monochlorobimane (MCB) readily crosses cell membranes and only becomes fluorescent after reaction with thiol groups. When cells are incubated with MCB at 37°C, there is a progressive increase in fluorescence at 420nm (after excitation at 360nm). The slope of the increase in median fluorescence over time gives an indirect measurement of the intracellular glutathione level in a homogeneous population of cells 8-10.

Separated cells were stimulated with a variety of reagents, as stated in chapter 7, and then mixed with MCB at a final concentration of 120mM. Cells were then analysed on the FACStar and data collected at 420nm over ten minutes; the slope of the curve for the increase in fluorescence was calculated. Hydrogen peroxide (20nM) and N acetyl cystine (50nM) were used as controls to decrease and raise intracellular thiol levels, respectively.

### 2.3.4. HIV gp120 Binding To CD4 Subsets.

These experiments were performed by a modification of the double staining method outlined above. PBMs (which had not been exposed to heparin) were incubated at  $10^6$  /ml in LCM containing recombinant gp120 (rgp120; Celltech, provided via the Medical Research Council's AIDS Directed Programme) 0.5µg/ml at either 37°C or 4°C. After variable periods of time, aliquots were removed and placed in large volumes of RPMI at 4°C. They were then stained with the cocktail of antibodies to select either CD4+ CD45RA+ or CD45RO+ lymphocytes, followed by rabbit anti - mouse FITC. gp120 was detected with biotinylated anti - gp120 antibody 55 (kindly provided by Dr Q Sattentau, for details on biotinylation, see below). After staining with AV - PE, cells were analysed on the FACScan. As in the calcium experiments above, FITC positive cells were gated out so that only the relevant CD4+ lymphocyte subset was studied.

### 2.3.5. Measurement Of TNF Receptors.

Cells were stimulated in 96 well plates ( $2 \times 10^5$  cells in a volume of 200µL RPMI with 10% FCS). Cells for stimulation with anti - CD3 were first incubated in saturating concentrations of UCHT1 (anti - CD3), washed 3 times and placed in 96 well flat bottom plates that had previously been treated with rabbit - anti - mouse - immunoglobulin (Sigma; 50µg/ml, pH 9.0 in bicarbonate buffer overnight at 4°C, then washed five times). Other cells were stimulated with Phorbol-12-Myristate-12-Acetate (PMA; Sigma) 5ng/ml, 1mM aminophylline (Sigma) in the presence or absence of cycloheximide 30µg/ml (Sigma) for varying times. Viability was greater than 90%, as assessed by Trypan Blue exclusion, after these procedures.

Cells were stained for expression of MHC Class II and IL2R $\alpha$  using FITC conjugated L243 and CD25 antibody (Becton Dickinson) and for TNF receptors p75 and p55 using antibodies Utr and Htr kindly provided by Roche<sup>11-14</sup>. Utr and Htr were biotinylated and used at 5 $\mu$ g/ml. FITC conjugated anti CD14 and biotinylated anti gp120 (mAb 55 from Quentin Sattentau) were used as a control for background staining. Cells labelled with biotinylated antibodies were stained with Av - PE.

## 2.4 Proliferation Assay.

For proliferation in response to phytohaemagglutinin (PHA), antigen or allogeneic stimulation, 200 $\mu$ l of cell suspension was placed in triplicate in round bottom 96 well plates at a concentration of 10<sup>6</sup>/ml in LCM. Adherent cells (allogeneic cells in the case of allogeneic stimulation) that had previously been incubated with mitomycin C (100 $\mu$ g/ml for 40 minutes) and then washed were added to a final concentration as shown in the text. In the case of stimulation with immobilised anti - CD3, cells were incubated with UCHT1 for 30 minutes, washed three times and then added to flat bottom wells in a 96 well plate which had previously been treated with rabbit anti mouse immunoglobulin as above.

Cells were incubated at 37°C in 5% CO<sub>2</sub> for between 3 and 5 days. 1 $\mu$ Ci tritiated thymidine was added (Amersham) and the cells cultured for another 8 hours. Incorporated thymidine was collected on filter discs by a semi automated harvester (Skatron), and radioactivity quantitated by a  $\beta$  emission reader (LKB).

## 2.5 Scatchard Analysis Of gp120 And Anti - CD4 mAb Binding.

Anti - CD4 mAb QS4120 Fab' fragments and rgp120 were labelled with <sup>125</sup>I by Dr A Pelchen Matthews, as described<sup>15</sup>. Anti - CD4 mAb QS4120 cleaved by pepsin to give monovalent fragments, and gp120 (Celltech for the Medical Research Council AIDS Directed Programme (MRC ADP)) were shown, by SDS-PAGE electrophoresis, to be more than 95% pure. These were incubated with Iodobeads (loaded with Na<sup>125</sup>I) and free iodine was removed with an Econo Pac column (BioRad). Specific activity was calculated by precipitating labelled protein with trichloroacetic acid and calculating the amount of radiation per milligram of precipitable protein. The specific activity (SA<sub>0</sub>) of Fab' Q4120 was 288 Ci/mmol and that of gp120 was 600 Ci/mmol, on the day of iodination.

Separated cells were washed twice in binding medium (RPMI without bicarbonate, 10mM HEPES, 0.2% BSA) and placed in V bottom 96 well plates (2x10<sup>5</sup> cells/well). Iodinated gp120 or Fab' QS4120 was added in 100 $\mu$ l 5%BSA in PBS at concentrations (as duplicates) ranging from 0.1 - 18nM. As a control for non specific binding, unlabelled QS4120 was added to some tubes at a saturating concentration of

30µg/ml. The cell suspension was incubated at 4°C for 2 hours or 37°C for 3 hours, with agitation.

Plates were centrifuged at 1000rpm for 2 minutes and 10µl of supernatant collected for estimation of free ligand. The cells were washed in binding medium and centrifuged through 1ml 5% BSA in PBS in an LP3 tube. The supernatant and BSA cushion were removed and the pellet (bound ligand) counted on a gamma counter.

Counts per minute were converted to Moles of ligand using the following formula:

$$\text{Moles ligand} = \text{cpm-background} / 2.22 \times 10^{12} \times \text{counter efficiency} \times \text{SA}_t$$

Where cpm = counts per minute

$$1 \text{ Ci} = 2.22 \times 10^{12} \text{ cpm}$$

$$\text{SA}_t = \text{Specific activity} = \text{SA}_0 \times 0.5^{\text{days elapsed since preparation}/60}$$

The bound (Moles of ligand in pellet) and free (Moles of ligand in supernatant) values for each point were plotted on a saturation graph. The regression value for non specific binding was calculated and subtracted from the total binding to give specific binding. Specific bound / free was calculated and plotted against Free ligand for each point. The intersect on the x axis gives the  $B_{\text{max}}$  (i.e. number of binding sites in pellet) and this divided by the number of cells in the pellet gives the  $B_{\text{max}}$  per cell. The reciprocal of the slope gives the  $K_d$  (i.e. affinity).

## 2.6. Electro Mobility Shift Assay Of NFκB.

### 2.6.1. Nuclear Protein Extraction.

To minimise artefactual *in vitro* activation of NFκB, cells were separated and activated and nuclear protein extracted within 24 hours. Additionally, culture was carried out in conditions of minimal *in vitro* activation; monocytes were removed at an early stage, FCS was replaced with autologous serum and LPS removed from culture media.

Nuclear material was prepared by a method modified from that of Dignam<sup>16</sup>, in which cells are lysed hypotonically, nuclear protein extracted by a high salt buffer and then re - equilibrated in an isotonic buffer since binding of proteins is inhibited by high salt concentrations.

One million cells were suspended in 10µl Lysis Buffer (for buffers see appendix III) and aspirated up and down ten times with a pipette. After incubation on ice for ten minutes, cell lysis was checked microscopically. Lysates were centrifuged in a cooled microfuge, for 5 minutes and the supernatant discarded. Ten microlitres of High Salt buffer was added to  $10^6$  cells and aspirated ten times through a pipette tip. Lysate was then placed on a rotator in a cold room for 15 minutes and centrifuged as before. The

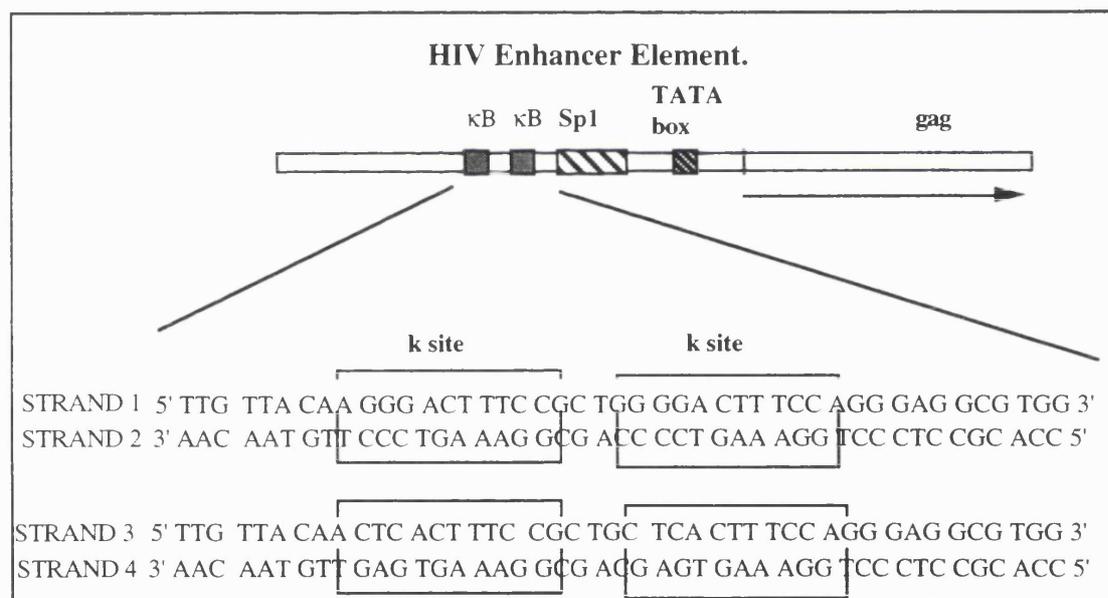
supernatant was collected and dialysed against 50 volumes of Equilibration buffer for an hour.

After dialysis, the amount of protein extracted was checked using the Bradford colorimetric assay (BioRad) according to the manufacturers instructions. The yield per  $10^6$  cells was 5-15 $\mu$ g. Extracts were frozen in liquid nitrogen until use.

### 2.6.2. Oligonucleotide Labelling.

Four oligonucleotides were synthesised by the MRC ADP, for use in the electro - mobility - shift - assay (EMSA). Strands 1 and 2 corresponded to the sense and anti sense strands respectively from -113 to 68 of the HIV 5' long terminal repeat (LTR) and have tandem repeats of the  $\kappa$ B motif <sup>17</sup>. In strands 3 and 4, a GGG (CCC in anti - sense strand) sequence was substituted with CTC (GAG), in each  $\kappa$ B motif. Strands 3 and 4 served as controls for non specific binding in the EMSA.

Figure 2.1. HIV Enhancer And Oligonucleotides Used In NF $\kappa$ B Assay.



### 2.6.3. Oligonucleotide/Protein Binding.

The oligonucleotides were 5' labelled by incubating 7.5pM of each with 15pM  $\gamma^{32}$ ATP (3,000 $\mu$ Ci/mmol; Amersham), 4U T4 polynucleotide kinase (Biolabs) in 50 $\mu$ l kinase buffer for 1 hour at 37 $^{\circ}$ C. The labelled oligonucleotides were separated from unbound  $\gamma^{32}$ ATP by passage through Sephadex G25 columns (Boehringer Mannheim). The overall efficiency of labelling for the 4 oligonucleotides was found to be 67.5%.

The sense and anti sense strands of the enhancer segment and the mutant were annealed by incubating at 87°C for 5 minutes in annealing buffer and allowing to cool slowly (the calculated melting temperature, from G/C ratio was 81°C).

#### 2.6.4. Electro Mobility Shift Assay.

The band shift assay was performed by the method of Singh<sup>18</sup>. This relies on the binding of proteins to the radio labelled  $\kappa$ B oligonucleotide retarding its transit through a polyacrylamide gel.

Eight micrograms of each nuclear protein extract was incubated for 30 minutes at room temperature with 4 $\mu$ g of poly(dI-dC) and 5 $\mu$ g salmon sperm DNA in 25 $\mu$ L of NF $\kappa$ B Binding Buffer. One nanogram of either native or mutated  $\gamma^{32}$ P labelled oligonucleotide was added and incubated for a further 30 minutes. In initial experiments a KBF1 peptide<sup>19</sup>, kindly provided by Dr Ron Hay, was used as a positive control. Briefly, the 35-381aa region of the KBF1 sequence<sup>19</sup> was expressed and purified by chromatography steps and by binding to DNA sepharose.

The reaction mixtures were run in 4% polyacrylamide gels in Low Ionic Strength Buffer in the absence of bromophenol blue (which can deNature NF $\kappa$ B), for 90 minutes at 11 V/cm, with buffer recirculation and cooling. The gels were then dried and exposed to X ray film at -70°C.

#### 2.7. Electrophoresis For DNA Fragmentation.

Following incubation with a variety of stimuli, cells were collected and washed twice in cold PBS prior to resuspension at 10<sup>6</sup> per 20 $\mu$ l in DNA Fragmentation Buffer with Proteinase K 500 $\mu$ g/ml. After incubation at 50°C for 1 hour, 10 $\mu$ l 0.5mg/ml RNase A was added (previously boiled to destroy any DNase activity). This was incubated for a further hour at 50°C and then heated to 70°C to inactivate both enzymes. Loading Buffer (10 $\mu$ l) was added and the resulting mixture loaded in to a 1% agarose gel prepared in Tris EDTA buffer containing 0.1mg/ml ethidium bromide, and then run at 40V for two hours.

#### 2.8. Polymerase Chain Reaction To Detect HIV After *In Vitro* Infection Of CD4+ T Cells Expressing CD45RA And CD45RO.

##### 2.8.1. Cell Infection.

A sub clone of the molecularly characterised HIV-1 strain HTLVIIIb/LAV called S1D6 was used in all experiments. This was inoculated into H9 cells during log growth and maintained by dividing and feeding twice a week for at least 30 passages. High titre stock was prepared by mixing infected and uninfected H9 cells at 2:1. These

cultures were shown to be free from Mycoplasma. Supernatant was harvested when twice daily examination showed maximal cytopathic effect (usually at four days).

Harvested supernatant was used fresh since freezing can result in a 100 fold loss of infectious titre. To check the infectious titre of the stock, it was titrated, in quadruplicate, onto MT2 cells at serial 10 fold dilution. These titrations were read at 5 days and so the multiplicity of infection (MOI) used in experimental infection was not available until after the experiments. The MOIs used in experimental infections ranged from 0.005 to 0.1.

Viral stock was centrifuged at 500g to remove cells and debris, filtered through 0.45µM filters and incubated with DNase (0.1U/ml, 37°C, 1 hour) to remove cellular and viral HIV DNA<sup>20</sup>. Any residual HIV genetic material was thus present as RNA.

Freshly separated populations of CD45RA+, CD45RO+ ClassII- or CD45RO+ Class II+ lymphocytes were re - suspended at 10<sup>6</sup>/ml in viral stock and incubated at 37°C for 3 hours with intermittent agitation. Cells were then washed three times in cold RPMI and then re - suspended in LCM at 10<sup>6</sup> /ml, with or without PMA and PHA, and incubated for a further 15 hours.

For each experiment, to control for DNA extraction and PCR and to make the assay semi - quantitative, dilutions of ACH2 cells were set up. ACH2 cells are A3.01 cells chronically infected with LAV/HTLVIII<sup>21</sup> contain one HIV DNA provirus per cell<sup>21</sup> and were obtained from the American Tissue Culture Collection (ATCC). ACH2 cells were diluted into fresh, uninfected CD4+ enriched cells immediately prior to DNA extraction.

In some experiments, the HPBALL T cell line was infected with HIV, washed five times, cultured and aliquots of supernatant were collected for p24 estimation by Elisa (Dupont). The technical cut off for this assay is 15pg; with this threshold, contaminating incoming p24 is not detected.

## 2.8.2. DNA Extraction

Cells were washed twice in cold PBS and then suspended at 10<sup>6</sup> cells in 100µl Promega Taq Polymerase Buffer and heated to 95°C for 1 hour to destroy residual DNase. Proteinase K was then added to a final concentration of 120µg/ml. The suspension was incubated at 50°C overnight after which it was heated to 90°C for an hour to inactivate protease. DNA was sheared by passing it through a p200 pipette tip 20 times, sonicated for 4 minutes and frozen until use.

## 2.8.3. Polymerase Chain Reaction.

Primers for HLA DQα were used as an internal standard; GH 26 and GH27 were synthesised by the MRC ADP and produce a 242 base pair amplificate<sup>25</sup>. The HIV primers used (M667 and M661) were derived from ARV sequences<sup>22</sup> but are conserved in LAV/HTLVIII<sup>23</sup>. These primers were designed to span the junction of the 5' LTR

and *gag*<sup>22</sup> and hence will only detect fully reverse transcribed provirus<sup>24</sup>. These primers were synthesised by Tib Molbiol (Berlin) and produce a 200 base pair product<sup>24</sup>.

M667; bases 496-516; Minus Strand

5' GGC TAA CTA GGG AAC CCA CTG 3'

Melting point; 59.5°C

M661; bases 695-672; Plus strand

5' CCT GCG TCG AGA GAG CTC CTC TGG 3'

Melting point; 66.6°C

Since DNA was extracted in polymerase buffer, it did not require purification and was loaded directly into the PCR mixture. DNA from  $2.5 \times 10^5$  cells, in 15µL buffer, containing about 1µg DNA was used in each PCR reaction. The PCR reaction mixture was optimised, in initial experiments, using conditions shown in appendix III. Reagents and DNA sample were made up to 50µL in Eppendorf tubes, overlaid with mineral oil and the tubes placed in a thermocycler. To maximise sensitivity, a “hot start” technique was used whereby the reaction mixture was taken to 95°C for 10 minutes and then lowered to 85°C before adding Promega Taq Polymerase (4U) under the oil. This inactivates remaining DNase and Proteinase K and prevents incorrect extension of malaligned primers. Following this, the reaction went through 35 cycles as follows;

Melting	95.5°C	1 minute
Annealing	60°C	1 minute
Extension	72°C	1 minute

with a final period of 10 minutes extension at 72°C.

On completion of PCR 25µL aliquots of product were mixed with 5µL loading buffer (30% glycerol, 0.25% bromophenol blue) and loaded onto an agarose gel (3% LMP Agarose, BioRad, TBE, 0.1% ethidium bromide). The gels were run for an hour at 100V and inspected with ultraviolet transillumination.

## Yellow Fever Virus Infection.

Separated CD45RA+ and CD45RO+ CD4+ T cells or H9 cells were incubated for 1 hour with yellow fever virus 17B at an MOI of 0.1. Cells were washed 3 times and then cultured for varying times before aliquots of supernatant were taken for virus titration. Supernatant was cultured at triplicate  $\log_{10}$  dilutions on monkey kidney cells which were checked daily for plaque formation. The infective titre was then calculated.

## 2.10. Appendix I. Monoclonal Antibody Production, Purification And Biotinylation.

Antibodies used in cell separation were used in large quantities and hence were produced from hybridoma supernatant grown in the laboratory. Hybridoma cells were provided by Peter Beverley and Quentin Sattentau (Tumour Immunology Unit, University College ,London Medical School), George Janossy (Royal Free Hospital, London) and the ATCC. Hybridoma cells were grown in LCM in 5% CO<sub>2</sub>. Supernatant was removed every two to three days and centrifuged at 400g prior to freezing or purification.

Anti - envelope 55, UCHL1, SN130, and UCHT1 and QS4120 antibodies were purified in preparation for biotinylation (or adherence to plastic, in the case of UCHT1). Hybridoma supernatant was passed over a Protein A Sepharose Column and equilibrated at pH 8 with phosphate buffer. After 24 hours of recirculation, the column was washed with more pH 8 phosphate buffer and the antibodies eluted with citrate buffer at the appropriate pH for the antibody isotype (pH 6.0 for IgG1 (UCHT1, SN130, QS4120), pH4.5 for IgG2a (,UCHL1, anti - env 55). Eluate pH was immediately restored to pH 7 with Tris and the concentrated over an Amicon YM10 filter to at least 5mg/ml.

In addition to the above antibodies, utr and htr (obtained purified) were also biotinylated, for use in double staining. Purified antibodies were dialysed against NaHCO<sub>3</sub> pH8.6 overnight before adding biotin-X-N-hydroxysuccinimide (Cambridge Biosciences) (stock 1mg/ml in di - methyl - sulphoxide) to a final concentration of 100µg/ml and rotating at room temperature for 4 hours. After overnight dialysis against PBS containing 0.1% sodium azide, the biotinylated antibodies were titrated on appropriate cells.

## Appendix II. Monoclonal Antibodies Used.

Antibody	Cluster of differentiation	Originator	Ref
UCHT1	CD3	Peter Beverley	26
QS4120	CD4 (V1 domain, gp120 binding site)	Quentin Sattentau	27
Leu 3A	CD4 (V1 domain, CDR 1 and 2)	Becton Dickinson	28
72G4	CD4 (V1 domain, gp120 binding loop)	MRC ADP	
L104	CD4 V1 domain CDR3 region	Becton Dickinson	29
MT151	CD4 (V1 domain, CDR1 and 3)	P. Reiber and G. Reithmuller	30
QS425	CD4 (third domain, fusion inducing region)	Quentin Sattentau	27
MT429	CD4 (fourth domain)	P. Reiber and G. Reithmuller	
UCHT4	CD8	Peter Beverley	
OKM1	CD11b	ATCC	
UCHM1	CD14	Peter Beverley	31
MHM23	CD18 (LFA-1 $\beta$ )	Dako	32
BU12	CD19	N. Ling	33
UCHL1	CD45RO	Peter Beverley	34
SN130	CD45RA	George Janossy	35
Anti gp120 55	HIV gp120	Q. Sattentau / MRC ADP.	
htr	55kD TNF receptor	Brockhaus, Hoffman La Roche, Switzerland.	13 12
utr	75kD TNF receptor	Brockhaus, Hoffman La Roche, Switzerland.	13 12
L243	HLA Class II	ATCC	36

## Appendix III. Buffers

### Nuclear Protein Extraction Buffers

#### Lysis Buffer;

1.5 mM MgCl<sub>2</sub>  
0.5 mM dithiothreitol (DTT)  
10 mM HEPES, (pH 7.9)  
10 mM KCl  
0.5 mM Phenoxy methylsulfonylfluoride (PMSF)  
0.1% NP40

#### Equilibration Buffer;

20mM HEPES  
5mM DTT  
20% glycerol  
5mM KCl  
0.5mM PMSF

#### High Salt Buffer;

0.5mM DTT  
0.3M HEPES, (pH 7.9)  
1.4 M KCl  
0.5mM PMSF

DTT and PMSF were added fresh each time.

### Oligonucleotide Labelling And Annealing Buffers

#### x10 T4 Kinase Buffer;

500mM imidazole. Cl (pH 6.4)  
180mM MgCl<sub>2</sub>  
50mM DTT  
1mM EDTA  
1mM spermidine  
Adenosine di phosphate 1mM  
Adenosine tri phosphate 50nM

#### Annealing Buffer;

0.1M NaCl  
10mM Tris/HCl (pH 7.8)  
1mM EDTA

### Electromobility Band Shift Buffers

#### NFκB Binding Buffer;

4mM GTP,  
50mM NaCl  
Leupeptin and pepstatin A 2μg/ml  
10mM Tris-HCl (pH 7.5)  
1mM EDTA  
1mM DTT  
5% glycerol.

#### Low Ionic Strength Buffer;

3.3mM Sodium Acetate  
6.7. mM Tris/HCl (pH 7.5)  
1mM EDTA.

## DNA Fragmentation Buffers

### Lysis buffer;

10mM EDTA

0.5% sodium lauryl sarkosinate

50mM Tris/HCl pH8.0

### Tris EDTA buffer;

2mM EDTA

80mM Tris

### Loading buffer;

1% low temperature gelling agarose

40% sucrose

10mM EDTA

0.25% bromophenol blue

## Optimised Polymerase Chain Reaction Buffer

KCl

50mM

Tris HCl pH9.0

10mM

Triton X 100

0.1%

Mg<sup>++</sup>

2mM

HIV primers

50nM

HLA DQ $\alpha$  primers

5nM

dNTPs

100 $\mu$ M

25 $\mu$ l

Extracted DNA

25 $\mu$ l

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# Chapter 3. CD4 Expression And Gp120 Binding On CD45RA+ And CD45RO+ Lymphocytes.

## 3.1. Introduction.

The binding of gp120 HIV envelope glycoprotein to CD4 is central to the pathogenesis of HIV infection since it is required for viral attachment, fusion<sup>1</sup> and, subsequently, syncytium formation<sup>2</sup>. Gp120 binding also induces abnormal signals in T cells which may be important in virus infection and syncytium formation (see chapter 5) and, if they occur after binding of soluble gp120, may be an important cause of the immunosuppression of AIDS (see chapter 4).

Because the ramifications of gp120 binding are so far reaching, it was important to determine that both CD45RA+ or CD45RO+ CD4+ lymphocytes are able to bind gp120 equally well.

### 3.1.1 gp120

The *env* protein is initially transcribed as a precursor, gp160, and is then cleaved to two sub units, gp41 and gp120 before delivery to the cell surface. Gp41 is located in the cell membrane and is non covalently associated with gp120. Gp41/120 complexes appear to polymerize<sup>3</sup> and it appears that the basic unit is a dimer which can also dimerize to form a tetramer<sup>4</sup>. The dimer building blocks are held together non covalently by association between gp41<sup>4</sup> and presented as spikes on the virus surface<sup>5</sup>.

The HIV envelope glycoprotein gp120 plays a key role in virus binding and viral fusion/syncytia formation. Crystallography data are not yet available on gp120 and information on functional domains is derived from experiments in which the amino acid sequence is mutated. The carboxy terminus of gp120 is involved in interaction with the HIV fusion protein, gp41<sup>6</sup>. Domains scattered across gp120, presumably brought together in folding, form the CD4 binding site (V3, C3, and C4)<sup>7</sup>, whilst the V1, V2, V3, C2 domains are involved in syncytium formation<sup>8</sup>. Changes in these latter domains confer the ability of HIV strains to form syncytia *in vitro*.

Following binding of gp120 to CD4, conformational changes take place in gp120 which expose the V3 loop of gp120, promote gp120 shedding from gp41, and make V3 vulnerable to proteolysis<sup>1</sup>. Whether shedding and/or proteolysis of gp120 are important *in vivo* is unclear (reviewed in<sup>5,9</sup>). A T cell activation molecule, CD26 (peptidyl peptidase IV) has been proposed as a co - factor for infection on the basis that it may cleave the V3 loop as a prerequisite for infection<sup>10</sup>, although the evidence for this remains highly controversial (see next chapter). Gp120 is nonetheless dissociated from

gp41, allowing the latter molecule to interact with the cell surface. To date, no ligand for gp41 has been identified.

These changes in the conformation in gp120 appear to occur synchronously with similar changes in CD4. This phase of the interaction may take up to two hours to be completed, after viral binding <sup>11</sup>.

Oligosaccharide residues on gp120, which are responsible for more than 50% of the molecular weight, appear to be involved in both gp120/CD4 binding and in syncytium formation/fusion (reviewed in <sup>12</sup>). The glucosidase inhibitor castanospermine, which is being tested clinically, results in gp120 glycosylation changes which do not reduce binding, but diminish infectivity. The CD4 binding and syncytium/virus fusion determining roles of gp120 hence appear to be dissociated. This dissociation is mirrored in CD4, as discussed below.

A recent finding is that MHC Class II<sup>5</sup> and other T cell surface molecules<sup>13</sup> appear to be concentrated in HIV envelope. Whether these are involved with gp120 binding in some way is unclear and is discussed further in chapter 5.

### 3.1.2. Different Regions Of CD4 Have Distinct Roles In The Gp120 Interaction.

CD4 is the major receptor for HIV<sup>14</sup>. Although other surface molecules may bind gp120, infection only takes place when CD4 is also expressed. For example, V<sub>H</sub>3 immunoglobulin domain has high affinity binding to gp120<sup>15</sup>, but B cells are not infected with HIV *in vivo*<sup>16</sup>.

The initial discovery that some anti - CD4 mAbs block HIV infection of lymphocytes <sup>14</sup> was supported by the finding that soluble CD4 <sup>17</sup> had similar effects. Transfection of CD4 into epithelial cells made them permissive for infection<sup>18</sup>, confirming that CD4 is the HIV receptor. Although the presence of CD4 is prerequisite for HIV infection of cells, it is not sufficient for this process; animal cells transfected with CD4 will not support HIV infection<sup>19</sup>.

Studies with mAbs against defined epitopes of CD4 subsequently showed that the V1 domain of the CD4 molecule is the binding site for gp120<sup>20, 21</sup>. In the course of these studies it became clear that there were two groups of non - cross blocking anti - V1 mAbs, which inhibited either gp120 binding or syncytia formation, suggesting that two separate sites on V1 were important for infection. The first of three strands of data showed that an antibody which binds the CDR3 region of V1 inhibited subsequent virus fusion and syncytium formation but did not block binding of gp120<sup>22</sup>. A second anti - V1 antibody inhibited infection of cells with cell free virus and syncytium formation when added up to an hour after adding virus or infected cells. This antibody did not displace HIV from cells after mixing and so conformational changes in V1 induced by this antibody may impair both fusion and syncytium formation after binding <sup>11</sup>. Finally, cell lines with mutations in CD4 did not become infected with HIV, despite binding gp120 adequately<sup>23</sup>. These cell lines also showed reduced syncytium formation with

cells transfected with gp160, implying the same defect could impair viral fusion and subsequent syncytium formation. Hence, the gp120 binding and syncytium forming regions of V1 are functionally separable.

X ray crystallography data resolved the configuration of the CDR2 and CDR3 regions in V1 by showing these regions lie on opposite faces of the V1 domain. The existence of a CDR2 ridge, suggested by these data<sup>24, 25</sup>, is also consistent with the difficulties found in trying to raise anti - idiotypic antibodies against anti-CD4 to block gp120 binding; the CDR2 ridge fits into a deep groove on gp120, which is inaccessible to antibody binding<sup>26</sup>. A hydrophobic phenyl alanine residue at position 43 in CDR2, surrounded by positively charged amino acids, seems to constitute the region most likely to fit the putative groove in gp120<sup>27</sup>.

The model that arises from these data is that the CDR2 ridge binds with high affinity onto gp120 whilst CDR3 is more involved in fusion. The role of CDR3 in fusion is discussed in chapter 5.

The aims of the experiments presented in this chapter were to study the expression of CD4 on unstimulated CD45RA+ and CD45RO+ CD4+ lymphocytes and their ability to bind gp120 as a prelude to studying *in vitro* infection and gp120 signal induction. Cells were unstimulated, in so far as the small proportion of circulating CD4+ lymphocytes expressing MHC Class II<sup>28</sup> were removed during the separation procedure.

## 3.2 Results

Cell populations throughout the experiments described in this thesis were prepared by physical negative selection or counter - staining and flow cytometry. Cell populations were routinely checked for purity and were > 95% CD4+ and > 90% CD45RA+ or CD45RO+. To avoid confusion populations are referred to as CD45RA+ (i.e. CD8- CD14- CD19- CD11b- MHC Class II-, CD45RO-) or CD45RO+ (i.e. CD8- CD14- CD19- CD11b- MHC Class II-, CD45RA-).

### 3.2.1 CD4 Expression Is The Same On CD45RA+ And CD45RO+ Cells.

The expression of CD4 epitopes on CD45RA+ and RO+ cells has not previously been systematically investigated. This molecule could conceivably be expressed differently on these populations, hence affecting gp120 binding.

Expression of a number of CD4 epitopes was assessed by flow cytometry. Data from 4 experiments were collected. Fluorescence histograms showed normal distribution with little spread, so mean fluorescence was measured. The average of the 4 means is shown in Table 3.1 and significance was tested with a 2 tailed paired T test (T value and p).

**Table 3.1. CD4 Epitope Expression On CD45RA+ and CD45RO+ CD4+ Lymphocytes.**

Antibody	CD45RO+	CD45RA+	T, p	CD4 Epitope
Leu 3A	616 (55)*	599 (72)	-1.8, 0.094	V1 domain, CDR 1 and 2 <sup>20</sup>
QS4120	498 (31)	475 (56)	-1.2, 0.36	V1 domain, CDR2 <sup>29</sup>
72G4	552 (111)	530 (121)	-2.6, 0.23	V1 domain, CDR2
L104	632 (97)	597 (98)	-4.6, 0.08	V1 domain CDR3 <sup>22</sup>
MT151	590 (87)	544 (97)	-3.2, 0.09	V1 domain, CDR1 and 3 <sup>30</sup>
QS425	463 (52)	447 (47)	-0.44, 0.70	V3, fusion inducing region <sup>29</sup>
MT429	497 (76)	477 (93)	-0.03, 0.98	V4

\*Mean fluorescence (sd).

The data in table 3.1 show each CD4 epitope is present in similar quantities on CD45RA+ and CD45RO+ cells. The slight increase in fluorescence on the CD45RO+ cells is consistent with the slightly larger size of these cells<sup>28</sup>.

### 3.2.2. Kinetics Of Binding Of gp120 Onto CD45RA- And CD45RO- CD4+ Cells Is Identical.

Binding of gp120 to CD4 is a dynamic process involving gp120/CD4 encounter, stable complex formation and endocytosis. In these experiments, non adherent mononuclear cells were exposed to gp120 at 5µg/ml 37°C or 4°C for various times. The gp120 preparation used, made by Celltech for the MRC ADP, was produced in Chinese hamster ovary cells and purified by immunoaffinity chromatography. Chromatography, done by Celltech, showed that 95% of the protein content was in a 120 K<sub>d</sub> band, suggesting high purity and full glycosylation (since more than 50% of the molecular weight of gp120 corresponds to sugar residues). After gp120 incubation, cells were washed extensively in chilled medium to prevent endocytosis and then stained to exclude all non CD4+ T cells and either CD45RA or CD45RO (see section 2.3.2.). A biotinylated anti gp120 antibody detected bound gp120. Two colour immunofluorescence was carried out to analyse gp120 binding in the negatively selected (not FITC labelled) cells.

Median PE fluorescence intensity data were collected with the FACS FL2 channel set on logarithmic mode. Distribution of PE fluorescence was not normally distributed and so median fluorescence values were analysed. Data shown in figures 3.2.1. and 3.2.2. have had baseline binding, i.e. cells unexposed to gp120, subtracted first.

Figure 3.2.1. Gp120 Binding To CD45RA+ And CD45RO+ Cells At 37°C.

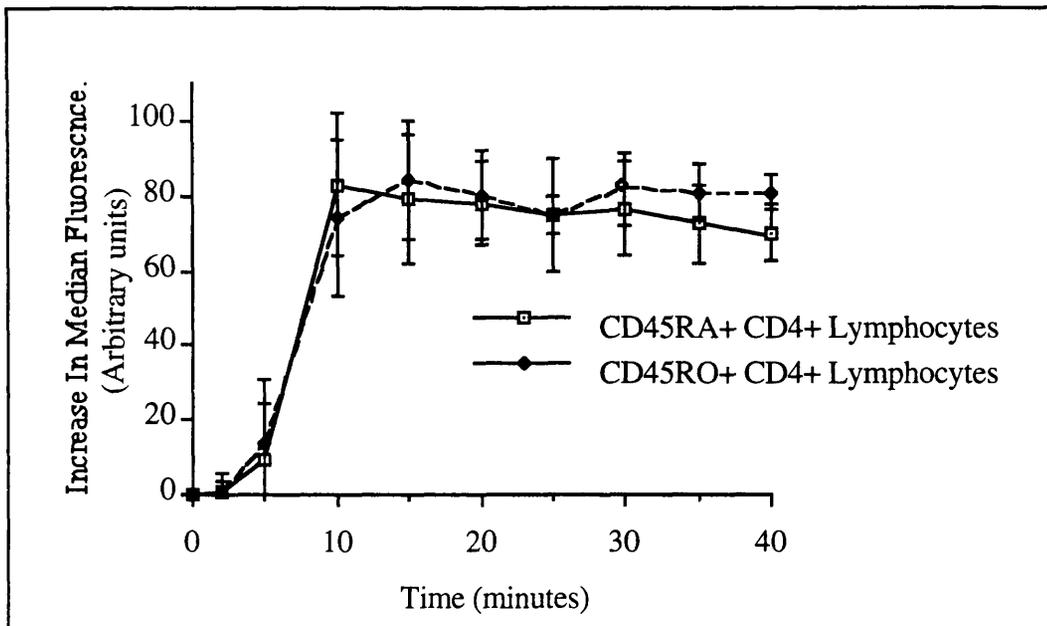
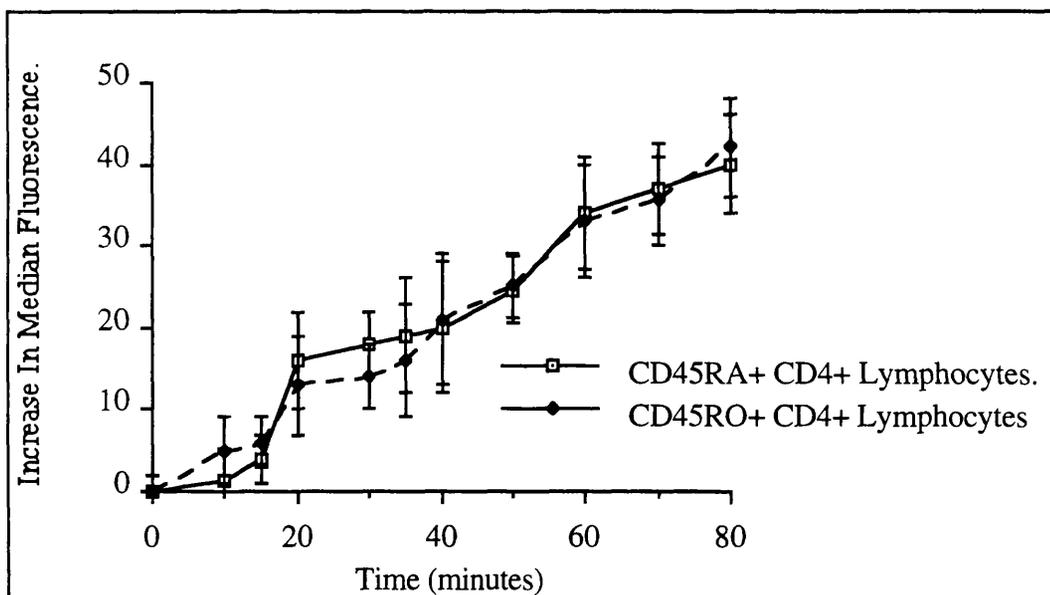


Figure 3.2.2. Gp120 Binding To CD45RA+ And CD45RO+ Cells At 4°C.



The gp120 binding kinetics at 37°C and 4°C are shown in figures 3.2.1. and 3.2.2. and represent median data and standard deviations from 6 experiments. At 37°C, binding is equally rapid in both populations and saturates after about 10 minutes. Both populations bind approximately equal amounts of gp120. The percentage of CD4+ cells in the CD8- CD14- CD19- CD11b- MHC Class II-population was checked using CD4AvPE. In each experiment the percentage of cells expressing CD4 was the same as the percentage of cells binding gp120 (not shown), suggesting all CD4+ cells were

binding gp120. After 10 minutes at 37°C the CD45RO+ population showed a slight trend to accumulate surface gp120, whilst the CD45RA population showed decreasing surface gp120. This is discussed further in the next chapter. Neither of these trends were significant.

Both populations bind gp120 equally slowly at 4°C. Saturation was not achieved in 80 minutes of incubation.

### 3.2.3. Scatchard Analysis Of Binding Of <sup>125</sup>I Labelled gp120 And QS4120 fab' Fragments To CD4+ CD45RA+ And CD4+ CD45RO+ Lymphocytes.

Scatchard analysis was carried out to quantify the affinity of gp120 for CD4 and confirm that the number of CD4 molecules available for gp120 binding was the same in the two populations of cells.

Conventional Scatchard analysis<sup>31</sup> of monoclonal antibody Fab' fragments to CD4 is straightforward since saturation occurs rapidly at 4°C without endocytosis complicating the assay. Although two major requirements for Scatchard analysis of gp120 binding were met, namely the gp120 was shown to be pure and the specific activity per gram of protein (and hence Mole of ligand) was known, gp120 binds CD4 only very slowly at 4°C (see above data and <sup>2</sup>) and stimulates down - regulation of CD4 at 37°C (see chapter 4) making formal analysis of the number of gp120 binding sites and their affinity difficult. The strategy adopted here was to first assess the number of binding sites for fab' fragments of the CDR2 antibody QS4120, which saturates at 4°C. Steady state dissociation constant ( $K_{ss}$ ), a value determined by association, dissociation and endocytosis<sup>5</sup>, for gp120 after 3 hours at 37°C and the comparative dissociation constant for both populations at 4°C, accepting saturation may not have occurred, were next evaluated. The number of binding sites at ( $K_d$ ) was assessed at both temperatures.

The Scatchard plots for QS4120 fab' and gp120 binding at 4°C and gp120 binding at 37°C are shown in figure 3.2.3. - 3.2.8. In each case the reciprocal of the slope is equal to the  $K_d$  of the reaction. The number of binding sites per cell ( $B_{max}$ ) is derived by dividing the x intercept by the number of cells per pellet, which was different for each population. These data are shown in table 3.2.

**Table 3.2.  $B_{max}$  And  $K_d$  For Q4120 And gp120 On CD4+ CD45RA+ And CD4+ CD45RO+ Lymphocytes.**

	$K_d$ QS4120 (nM)	$B_{max}$ QS4120	$K_d$ gp120 4°C	$B_{max}$ gp120 4°C	$K_{ss}$ gp120 37°C	$B_{max}$ gp120 37°C
CD45RA+	1.43	55000	8.71	2590	.77	2620
CD45RO+	1.29	46000	12.1	3850	1.2	3600



Figure 3.2.3. Scatchard Plot Of QS4120 Binding At 40C To CD45RA+ CD4+ Lymphocytes.

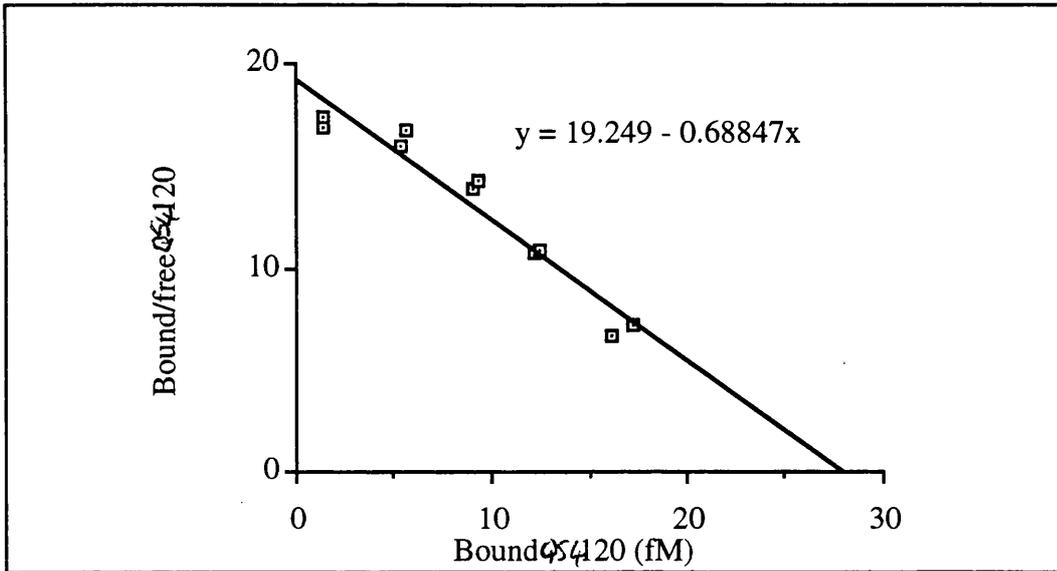


Figure 3.2.4. Scatchard Plot Of QS4120 Binding At 40C To CD45RO+ CD4+ Lymphocytes.

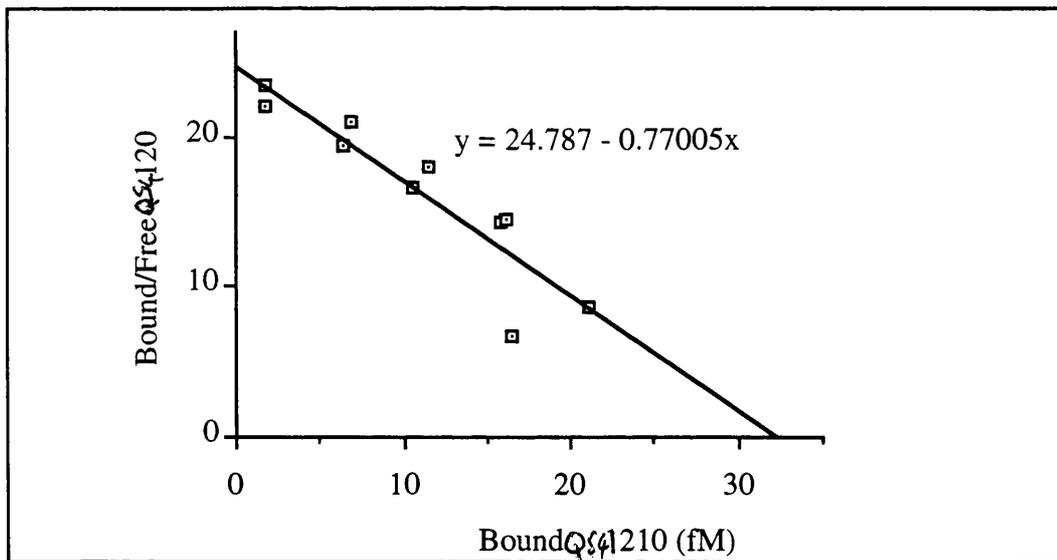


Figure 3.2.5. Scatchard Plot Of gp120 Binding At 4°C To CD45RA+ CD4+ Lymphocytes.

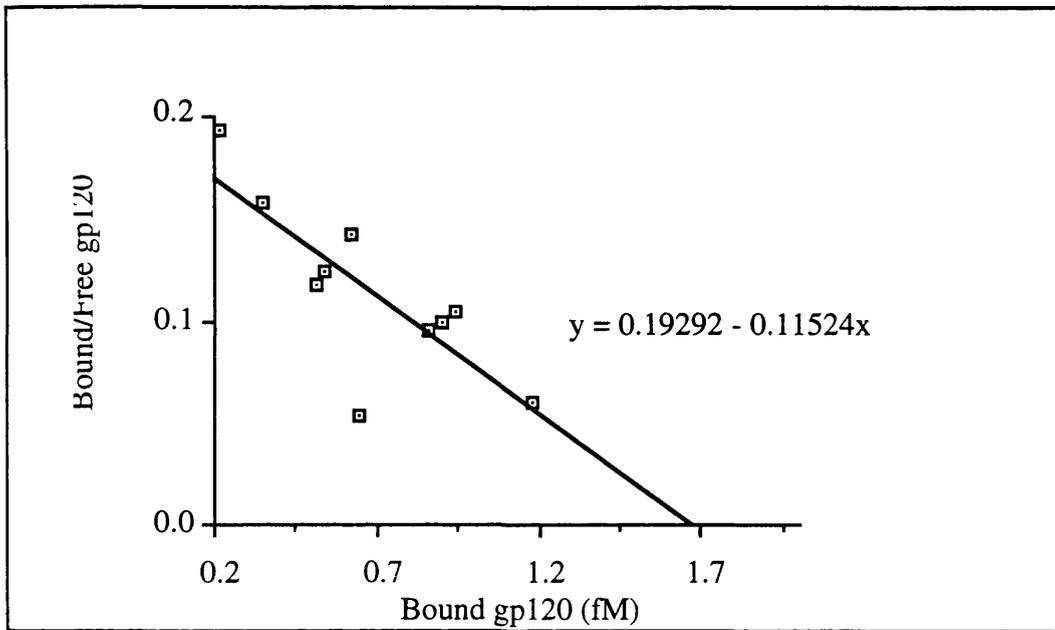


Figure 3.2.6. Scatchard Plot Of gp120 Binding At 4°C To CD45RO+ CD4+ Lymphocytes.

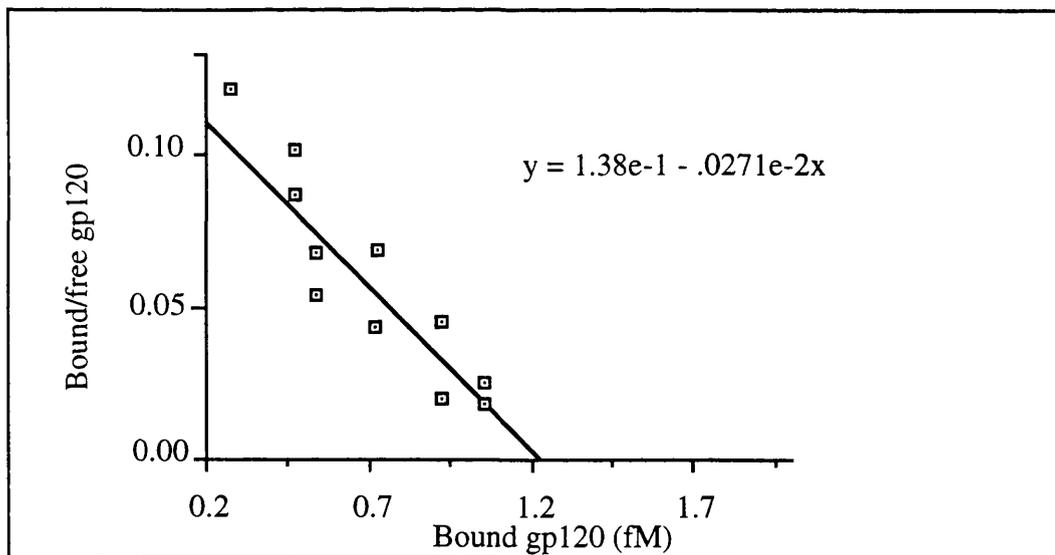


Figure 3.2.7. Scatchard Plot Of gp120 Binding At 37°C To CD45RA+ CD4+ Lymphocytes.

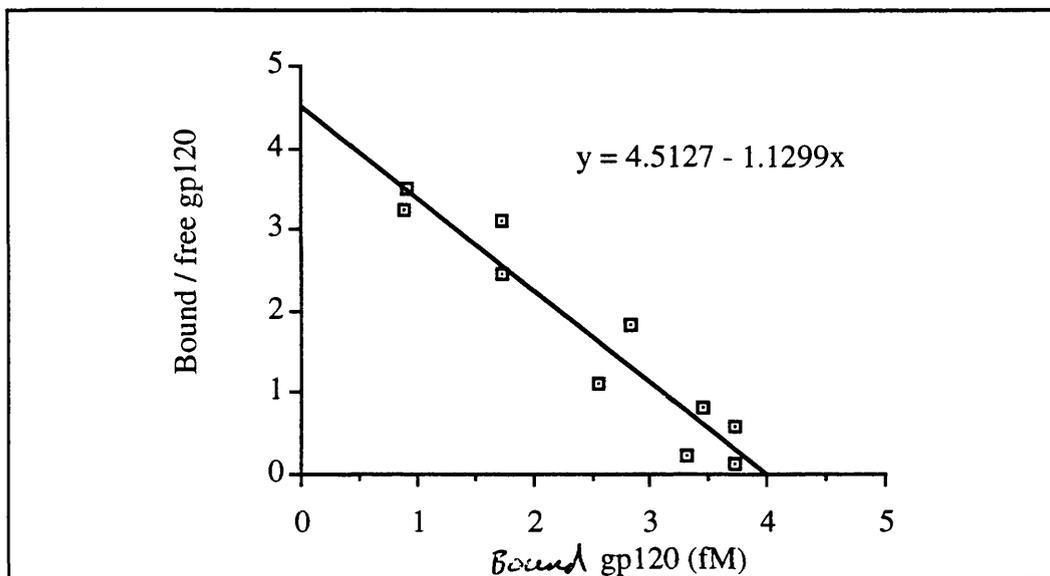
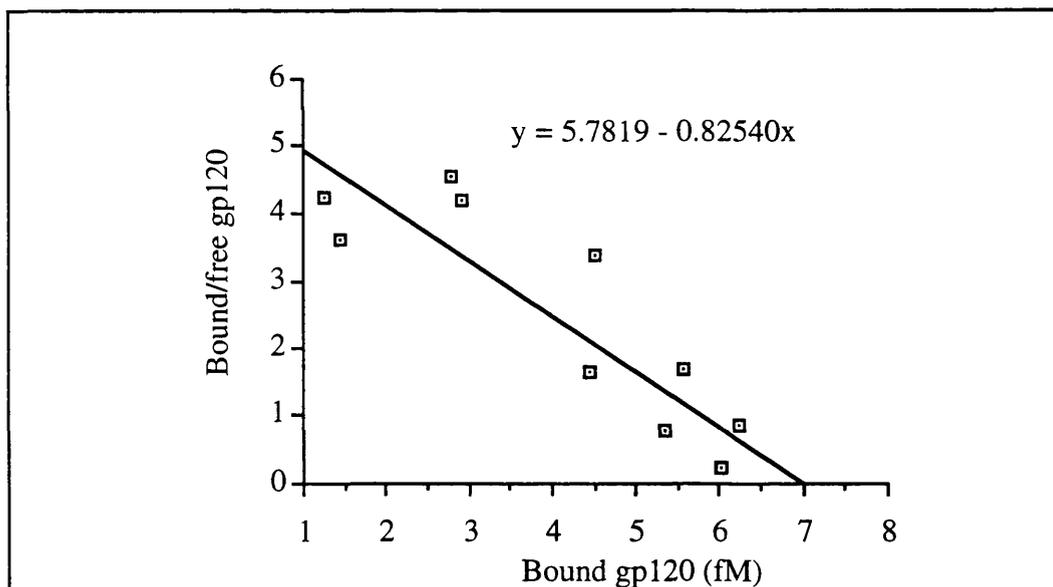


Figure 3.2.8. Scatchard Plot Of gp120 Binding At 37°C To CD45RO+ CD4+ Lymphocytes.



### 3.3 Discussion.

The data from the Scatchard analysis of anti - CD4 binding, shown in figures 3.3 and 3.4 and Table 3.2, confirm that both populations have very similar numbers of surface CD4 molecules, as previously shown (unpublished data in 44). The figure given here, of 50,000 per cell is close to that previously published<sup>32</sup>. Along with the flow cytometry data shown in table 3.1, this indicates that CD4 expression is similar in both CD45RA+ and CD45RO+ lymphocytes.

A number of surface molecules are up - regulated when CD4+ lymphocytes switch CD45 isoform expression from RA to RO, including CD29, CD58/LFA-3, CD18/LFA-1, CD2, CD54/ICAM and MHC Class II<sup>28, 33</sup>. These are adhesion molecules in the sense that they promote cellular interactions during antigen presentation and several can also transmit co - stimulatory signals. The up - regulation of these molecules is necessary for the hyper - responsive state of CD45RO+ cells<sup>34</sup> (see section 1.4.3. on hyper - responsiveness). CD4 shares some features of these accessory molecules; it ligates MHC Class II and has a co - stimulatory role in T cell activation<sup>35</sup>. It may be, therefore, surprising that its expression is not increased on CD45RO+ cells. A possible explanation for the lack of up - regulation of CD4 with switching to CD45RO expression is that all CD4+ lymphocytes express enough of the molecule to associate with TcR/CD3. Since CD3 is not up - regulated in the switch from CD45RA to CD45RO<sup>28</sup>, presumably to avoid the reduction in specificity associated with increased avidity, there is no requirement to up - regulate CD4.

The panel of antibodies used shows that there are no qualitative differences in CD4 expression on CD45RA+ and RO+ cells, at least in epitopes important in the HIV life cycle. This is consistent with data showing no evidence of post - transcriptional, pre - translational regulation of CD4 in T cells<sup>36</sup>. The 2 N glycosylation sites on the proximal domains of CD4 represent potential for post - translational regulation, since cell surface molecules can be differentially glycosylated on CD45RA+ and RO+ cells<sup>37</sup>. However, glycosylation of CD4 does not affect HIV infection<sup>38</sup> and was not investigated in these experiments.

The CDR2 and CDR3 regions were expressed at similar levels on CD45RA+ and RO+ cells, taking into account the larger size of the latter. The V3 domain epitope, associated with a putative hinge region is expressed equally in both populations. The binding of QS425 to this epitope is reduced after gp120 binds to CDR2, presumably because of conformational changes<sup>29</sup>. This suggests that conformation of CD4 at a major site of flexibility is the same in both populations.

The flow cytometry data show that initial binding of gp120 occurs equally in CD45RA+ and CD45RO+ cells (see figures 3.1 and 3.2). Binding is much slower at 4°C and saturation does not appear to occur. Since this assay is only detecting surface and not endocytosed gp120, the more rapid accumulation of gp120 at 37°C can only be accounted for by differences in the thermodynamics of the CD4/gp120 reaction. This is

consistent with data showing a 4 - 9 fold decrease in apparent affinity of soluble gp120 for cell associated CD4 at 4°C<sup>39, 40</sup>. Similarly, intact HIV binds less readily to T cells<sup>41</sup> and soluble CD4 will not strip gp120 from intact virions at low temperatures<sup>1, 42</sup>. Thus, it has been suggested that gp120/CD4 binding involves temperature dependent conformational changes in CD4 and/or gp120<sup>1</sup>.

Conformational changes may be required to transform an unstable "encounter complex" to a more stable structure<sup>5</sup> and have been identified in both gp120<sup>1</sup> and CD4<sup>29</sup> during interaction with one another. If conformational changes are required in CD4 for binding, they clearly take place equally well in CD45RA+ and RO+ cells, since both populations bind gp120 equally rapidly at 37°C and equally slowly at 4°C. These data are consistent with older data showing resting and stimulated T cells to bind HIV with similar kinetics<sup>41</sup>. Important conformational changes in CD4 also take place after gp120 binding, a topic addressed in the next two chapters.

Incubation at 4°C for 2 hours has been used to investigate the binding of gp120 to CD4<sup>39, 41</sup>, although subsequent data have shown that much longer incubations are required to reach equilibrium at this temperature<sup>27</sup>. The data presented here, derived after 2 hour incubations, can only therefore give apparent and relative information on gp120 binding between the 2 populations.

The data in table 3.2 show that the apparent dissociation constants ( $K_d$ , inversely related to affinity) of gp120 at 4°C is slightly lower for CD45RA+ than RO+ cells. These apparent  $K_d$  are rather higher than those previously published<sup>27</sup>. That the apparent  $K_d$  are close in both cell populations is not unexpected, since the data in table 3.1 show all the components of CD4 are present equally in both populations. The apparent  $K_{SS}$  data, after incubation at 37°C, are more difficult to interpret since they take into account the greater on and off rates at 37°C, the increased transition from putative "encounter complex" to stable structure and CD4/gp120 complexes which have been endocytosed and which will contribute to "bound" material, even after dissociation into intracellular sites. The  $K_{SS}$  is also slightly lower in CD45RA+ cells.

The apparent number of gp120 binding sites ( $B_{max}$ ) at 4°C and 37°C, determined by Scatchard analysis are slightly higher in CD45RO+ cells. This cannot be explained by a greater number of CD4 molecules on CD45RO+ cells, given the similar staining intensity for CD4 and the similar  $B_{max}$  for anti - CD4. Enhanced endocytosis does not appear to take place in the CD45RO+ population, since even at 4°C CD45RO+ cells have a greater  $B_{max}$  and the immunofluorescent data show a predominant loss of surface gp120 on CD45RA+ rather than CD45RO+ cells. It is not easy to offer an explanation for the greater number of gp120 binding sites on CD45RO+ cells in the presence of greater affinity and steady state binding, although one plausible reason is that although the affinity is greater on CD45RA+ cells, subsequent events in the development of a stable complex occur less readily, leading to a decreased number of bound gp120 molecules. This model may explain the decrease in surface gp120 in the immunofluorescent data on CD45RA+ cells at 37°C.

A major discrepancy in the gp120 Scatchard data presented is that there are fewer apparent binding sites for gp120 than for anti - CD4 fab' fragments. This is inconsistent with data showing that pre - incubation with gp120 can totally block anti - CD4 binding<sup>26, 43</sup> in flow cytometry experiments. The most likely explanation of the apparently lower  $B_{max}$  for gp120 is that although the determination of the specific activity of iodinated gp120 was correct, iodination of tyrosine residues in the C4 region of gp120<sup>7</sup> may have decreased CD4 binding. This could explain the  $B_{max}$  in these experiments being lower than that previously found, although the relative difference in  $B_{max}$  values for the CD45RA+ and RO+ populations remains valid.

In conclusion, CD4 expression is quantitatively and qualitatively the same on CD45RA+ and CD45RO+ lymphocytes. It has been more difficult to explore the gp120/CD4 interaction on these cells, because of the complex thermodynamics and because of problems preparing an adequate gp120 reagent. It does appear that both populations bind gp120 well, with increased affinity in CD45RA+ cells, but that cells expressing CD45RO accumulate more surface gp120 than their CD45RA+ counterparts.

Having established this is possible to compare signals induced by gp120 and syncytium formation in CD45RA+ and CD45RO+ CD4+ lymphocytes.

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## Chapter 4. Signals Induced By gp120 Binding to CD4.

### 4.1.1. Changes Mediated By The gp120 CD4 Interaction.

Investigations have shown that gp120, can induce a wide variety of changes in T cell function. Changes in T cells after contact with gp120 may be important in viral entry and other steps in the viral life cycle or may contribute to the immunosuppression of HIV infection. Examples of the latter type of phenomenon, in which soluble gp120 reagents were used, are shown in table 4.1.

An important proviso to these *in vitro* experiments is that a wide variety of gp120 preparation and T cells types have been studied. The glycosylation pattern of gp120 derived from *bona fide* infected human cells, sgp120, is different from rgp120 expressed in insect or rodent cells. Sugar residues represent more than 50% of the molecular weight of gp120 and are important for both T cell stimulation and CD4 binding (reviewed in 35). Gp120 may dimerise in solution<sup>36</sup>, probably through sugar residues. Hence differences in gp120 preparations may explain some of the discrepancies in the published data<sup>30, 31</sup>.

Whether these *in vitro* phenomena contribute to *in vivo* pathogenesis is unclear. Free gp120 is found when HIV is cultured *in vitro* and originates from viruses budding from infected cells<sup>37</sup>. This may give rise to very high levels of gp120 in tissues where replication is active, such as lymph nodes<sup>38</sup>, but this has not been directly assessed. In serum of infected individuals gp120 has only been detected at low levels around 50ng/ml, well below the dissociation constant for binding to CD4<sup>39</sup>. However, gp120 is frequently present in immune complexes<sup>39</sup> which, through aggregation, may compensate for the low concentration and potentiate abnormalities<sup>11</sup>. Low levels of gp120 in serum may itself be a consequence of the high affinity interaction with CD4, with circulating gp120 rapidly binding to CD4+ lymphocytes, although it has not been possible to show this<sup>18</sup>.

Table 4.1. gp120 Effects On Lymphocytes.

Cell Type	gp120 Type	Activity
T cell clone, Jurkat fresh lymphocytes.	rgp120*, sgp120#	p56 <sup>Lck</sup> activation and degradation, and CD4 down-regulation <sup>1-5</sup>
T cell clone, PHA blasts.	rgp120 (+/-cross linking), sgp120	No phosphorylation signals detected or p56 <sup>Lck</sup> activity <sup>6-8</sup> .
T cell clone, fresh lymphocytes.	sgp120; rgp120	IP hydrolysis and calcium influx; inhibits CD3 mediated signals <sup>9-13</sup> .
T cell clone.	sgp120 rgp120 (+/- cross linking)	No calcium influx <sup>6,9</sup> .
T cell clone.	HIV	CD4 phosphorylation; PKC activity <sup>14</sup> .
Fresh lymphocytes T cell clone.	sgp120, Bgp120, gp120	Inhibition of PHA, antigen specific and CD3 induced proliferation <sup>9, 15-19</sup> .
Fresh lymphocytes	rgp120	Inhibition of anti - CD4 mediated migration <sup>5</sup> .
Murine T cells transfected with CD4.	rgp120	HLA DR binding blocked <sup>20, 21</sup> .
Fresh lymphocytes.	rgp120	TNF secretion <sup>22</sup>
Fresh lymphocytes, T cell clone.	rgp120	No IL1, IL6 or TNF secretion <sup>23</sup>
Fresh lymphocytes.	rgp120	Apoptosis <sup>24-26</sup>
Fresh lymphocytes.	HIV	Super - antigen activity <sup>27</sup> .
Fresh lymphocytes.	gp120 peptides	Allo - reactivity <sup>28, 29</sup> .
T cell clone.	sgp120	Antibody dependent cellular cytotoxicity <sup>18</sup> .
Monocytes.	sgp120; not rgp120	IL1 $\beta$ , TNF $\alpha$ , GMCSF, IL6 and prostaglandin secretion <sup>30-32</sup>
B cells	rgp120	Binding to V <sub>H</sub> 3 and immunoglobulin <sup>33</sup> and TNF secretion <sup>34</sup>

\*recombinant gp120; derived from Chinese hamster ovary cells.

#supernatant gp120; from naturally infected T cells.

Bgp120; baculovirus vector, insect cell expression system.

Gp120 that has bound onto CD4+ cells stimulates a number of responses in other cells, for example antibody dependent cellular cytotoxicity<sup>18</sup> or CD4+ MHC Class II restricted killing after intracellular processing (discussed in 18). Cell surface gp120 cross linked by antibody may also induce apoptosis<sup>26</sup>. Bound gp120 has some homology to HLA DR and may also stimulate an allogeneic response<sup>28, 29</sup> which could result in killing or non specific immune activation and a graft - versus - host - like - response<sup>40-42</sup>. A corollary of this hypothesis would be that *in utero* infection and subsequent tolerisation, or therapeutic immunosuppression would improve clinical outcome, which is not consistent with clinical experience<sup>43</sup>. Another hypothesis is that gp120 can act as a super - antigen<sup>27, 44</sup>, although there are no consistent changes in T cell receptor V $\beta$  gene usage<sup>45</sup>. Other data show that *in vitro* the inhibitory effects of gp120 (see below) outweigh the stimulatory effects<sup>19</sup>. CD4 is the ligand for a lymphocyte chemotactic factor and it is has been suggested that gp120 could interfere with this interaction<sup>5</sup>.

Extensive data suggest that gp120 acts directly on CD4+ T cells and monocytes to inhibit proliferation (see table 4.1). One explanation for the inhibitory effects of gp120 on antigen responses<sup>9, 18</sup> is that gp120 binds to CD4 with high affinity and interferes with the TcR/MHC Class II antigen interaction. It has been confirmed that such interference can take place<sup>20</sup> and since Class II ligation induces activation signals in antigen presenting B cells and monocytes<sup>46</sup> this may have implications for both T cells and APCs. However, simple interference with the Class II binding does not explain how signals stimulated by anti - CD3, which are independent of Class II, are inhibited and anti - CD4 antibodies can inhibit T cell stimulation in allogeneic responses whether or not they bind the MHC Class II recognition sites<sup>47</sup>.

Further possibilities are that gp120 delivers a "negative" signal to the T cell activation system or prevents the formation of the CD3/TcR/CD4 activation cluster complex<sup>47</sup>. The latter may be a possibility since multimeric gp120 aggregates CD4<sup>48</sup>; analogously, both F(ab) monomers and intact anti - CD4 can induce calcium signals, but only intact antibody can inhibit them<sup>13</sup>. These data infer that when CD4 is cross - linked, for example by gp120, it cannot take part in the activation complex.

Kornfeld and others<sup>9-13</sup> found that sgp120 can induce calcium influx in resting T cells<sup>10</sup>. Rgp120 does not always stimulate calcium influx itself, unless aggregated with immune serum, but can inhibit signals through the T cell receptor<sup>11</sup>. Because gp120 inhibits T cell responses at concentrations at which CD4 is not saturated<sup>18</sup> and not all CD4 molecules are cross - linked, it remains likely that negative signal delivery is the strongest effect of gp120. A proximal step in T cell activation, generation of inositol triphosphate from membrane phosphoinositides, is also stimulated by exposure to sgp120<sup>10</sup> or infection of cell lines with HIV<sup>49</sup>. Such an event, occurring even after exposure to non - saturating levels of gp120, may be involved in delivering the kind of negative signal observed.

In summary, there is little evidence that gp120 exerts stimulatory signals on T cells and, in general, effects appear to be inhibitory. Inhibitory effects of gp120 may be

mediated by physical interference with components of the T cell signalling mechanism or negative biochemical signalling.

#### 4.1.2. gp120 And p56<sup>Lck</sup>.

Investigation has focused on p56<sup>Lck</sup> because it associates with the ligand for gp120 and transmits very early activation signals. p56<sup>Lck</sup> has been shown to mediate tyrosine phosphorylation of cellular proteins after exposure of T cells to soluble gp120 or gp160<sup>3</sup>, cell free virus and infected cells expressing gp120<sup>50</sup>. Gp120 and gp160 have been shown to increase autophosphorylation and kinase activity of p56<sup>Lck</sup><sup>3</sup>. This activation does not require cross linking<sup>4</sup> and so presumably occurs because of conformational changes in CD4/p56<sup>Lck</sup> upon gp120/160 binding.

The cellular proteins phosphorylated by p56<sup>Lck</sup> after gp120/160 binding appear to be qualitatively<sup>2</sup> and quantitatively<sup>3</sup> different from those induced by anti - CD4 binding (even when the anti - CD4 antibody is against the gp120 binding site). For example, cdc2, a protein which inhibits cell cycling when phosphorylated, is phosphorylated after gp120 binding and its appearance coincides with the onset of syncytium formation<sup>50</sup>. The reasons for these differences in anti - CD4 and gp120 mediated signals are unclear.

Activation of p56<sup>Lck</sup> by gp120, like anti - CD4 alone, leads to its dissociation from CD4<sup>3</sup>, which in turn leads to CD4 down - regulation<sup>1, 3</sup>. p56<sup>Lck</sup> dissociated from CD4 is inaccessible to the signalling mechanism and may be vulnerable to intracellular degradation<sup>1</sup>. Hence p56<sup>Lck</sup>/CD4 dissociation may inhibit T cell activation, with apoptosis as a possible sequel<sup>24</sup>. Other studies which have failed to detect gp120 induced p56<sup>Lck</sup> activation (see Table 4.1), searched for p56<sup>Lck</sup> in anti - CD4 immunoprecipitates; p56<sup>Lck</sup>/CD4 dissociation may explain the negative results<sup>6-8</sup>.

These deleterious effects of CD4 binding by gp120 may occur because there is no concurrent CD3 stimulation. In one report<sup>4</sup>, gp120 induced p56<sup>Lck</sup> kinase activity was modified by simultaneous co - stimulation of CD3, although it was not determined whether this activity was still associated with CD4.

Hence gp120 can activate p56<sup>Lck</sup> and stimulate its dissociation from CD4, resulting in CD4 down - regulation and subsequent unresponsiveness to CD3 stimulation. When the tertiary structure of gp120 is appropriate, calcium influx and partial activation appears to occur; this may be mediated by CD4 cross linking.

A major part of the deleterious effects of gp120 appear to be mediated by p56<sup>Lck</sup> and this molecule is in turn regulated by CD45. Cells expressing different isoforms of CD45 respond differently to activation signals, most notably CD45RO+ cells proliferate better in response to soluble anti - CD3 stimulation<sup>51, 52</sup>; whether this is a consequence of the CD45 isoform itself or of other surface antigens up - regulated on the CD45RO+ cell is unclear. It is possible that CD45 isoforms modulate gp120 effects differently and that gp120 thus has variable effects on T cells, depending on the isoform expressed. The experiments in Chapter 3 show that the binding of gp120 to CD4+

CD45RA+ and CD45RO+ cells is very similar. Activation signals subsequent to gp120 binding may differ in these populations and the experiments in this chapter aim to investigate calcium influx and down - regulation of CD4, both consequences of p56<sup>Lck</sup> activation by gp120, in these populations.

## Results.

### 4.2.1. Changes In Intracellular Calcium After gp120 Binding In CD45RO+ And CD45RA+ T Cells.

The data in figures 4.1 - 4.3 are for negatively selected CD4+ CD45RA+ and CD4+ CD45RO+ populations, as described in 2.3.2. Briefly, cells were loaded with the calcium ionophore Indo 1 and then stained to exclude all non CD4+ T cells and either CD45RO or CD45RA. Median fluorescence ratio refers to the median of the ratios, for each event collected, of fluorescence at 480 and 400nm. This ratio is directly proportional to the absolute intracellular calcium concentration. The absolute value for intracellular calcium was not estimated.

Figure 4.1 shows changes in intracellular calcium concentration after stimulation with anti - CD3 and is typical for the three experiments performed. Antibody was dialysed against three changes of LCM and control was LCM with nothing added. These data support earlier studies which found that soluble, un - cross linked anti - CD3 can induce calcium influx <sup>53</sup>, which is more pronounced in CD45RO+ T cells<sup>54-56</sup>. The percentage of cells responding is similar in both populations (87% of CD45RO+ and 83% of CD45RA+ at 600 seconds), suggesting that the difference between the populations is the intensity of the calcium signal rather than the proportion of responding cells.

Figure 4.1 Calcium Influx After Anti - CD3 Stimulation.

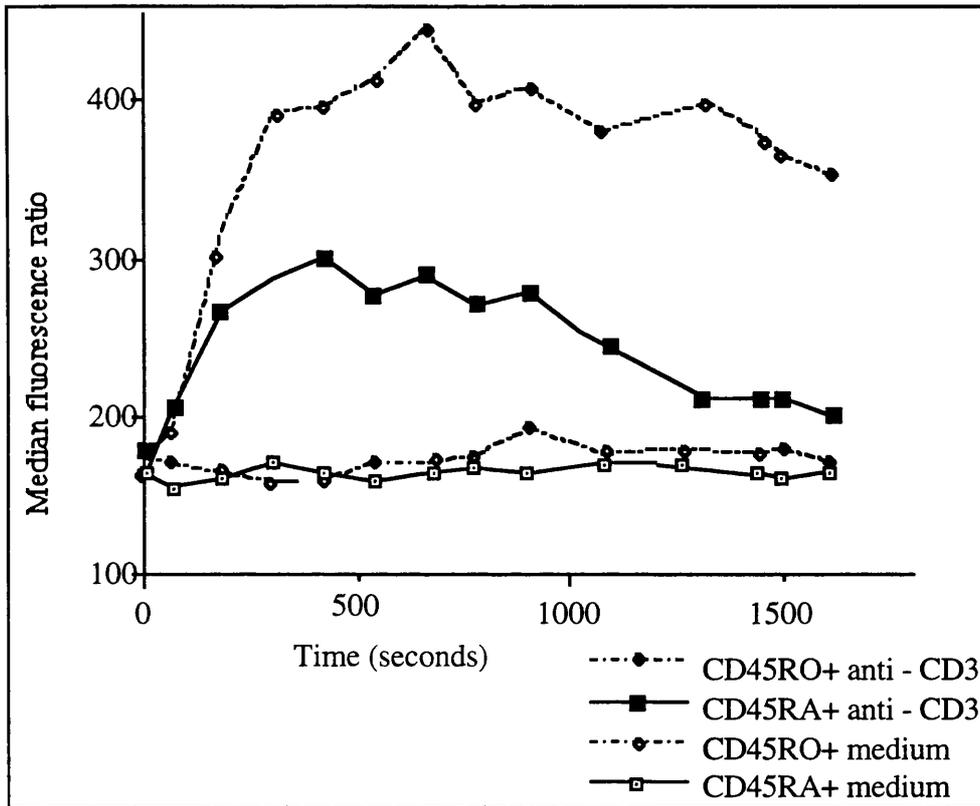
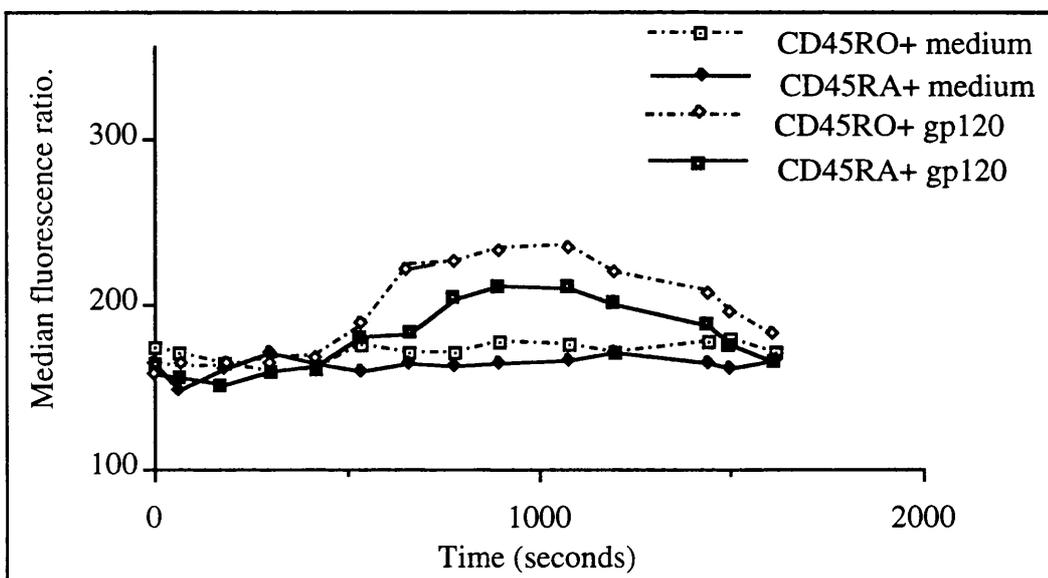


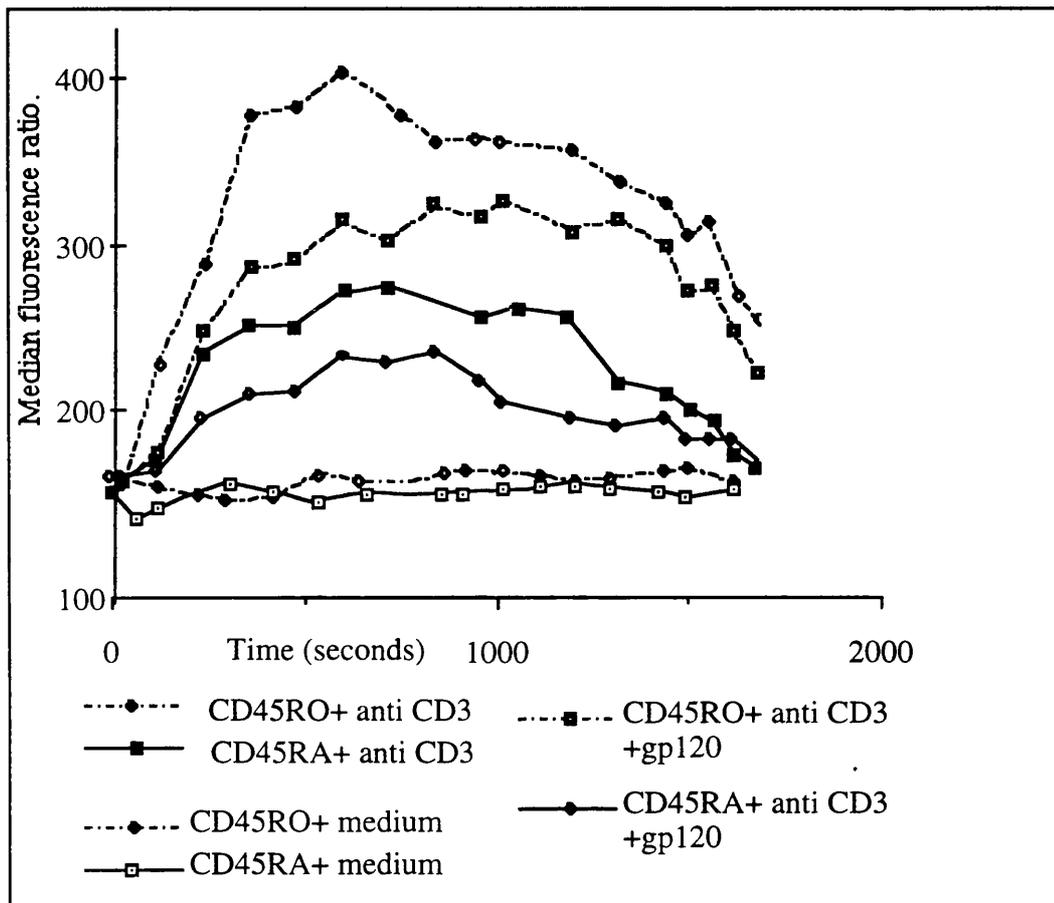
Figure 4.2 shows calcium influx after exposure to freshly dialysed rgp120 (5µg/ml) and represents data typical for 3 experiments. Both populations respond to gp120 although calcium influx is delayed and of much lower amplitude, compared to that obtained with anti - CD3, as previously noted<sup>13</sup>. CD45RO+ cells produced a response of slightly greater amplitude in all 3 experiments.

Figure 4.2. Calcium Influx After gp120 Stimulation.



When cells were mixed with *rgp120* (5 $\mu$ g/ml) or medium for 5 minutes at 37°C before exposure to anti - CD3 (Figure 4.3), *gp120* pre-treatment impairs calcium influx in both populations. As has been noted before, *gp120* can inhibit without the requirement for cross linking both in an antigen driven system<sup>9</sup> and when anti - CD3 is used<sup>57</sup>, although this is the first example of a recombinant glycoprotein doing so. The degree of CD3 signal suppression appears to be similar in both populations.

Figure 4.3 Anti - CD3 Stimulation After *gp120*



To see whether more proximal steps in the T cell activation pathway are affected by *gp120*, an attempt was made to study inositol polyphosphate hydrolysis after *gp120* stimulation. Cells were incubated in myo - 2(<sup>3</sup>H) - inositol using standard procedures<sup>9</sup>. Insufficient tracer was taken up to be able to identify any inositol metabolites, possibly because of the very low metabolic rate of these unstimulated T cells as previously noted<sup>13</sup>.

## 4.2.2. Modulation Of CD4 Expression In CD45RO+ And CD45RA+ T Cells.

Separated CD4+ CD45RA+ or CD45RO+ lymphocytes were cultured ( $10^6$ /ml in 12 well plates) at 37°C with dialysed gp120 (5 $\mu$ g/ml) or LCM alone. Aliquots of cells were taken at varying times and kept at 4°C until they were stained with MT429, an anti - CD4 antibody which does not compete with gp120<sup>58</sup> and hence allows determination of CD4 expression even after gp120 binding.

Figure 4.4 CD4 Down - Regulation After gp120

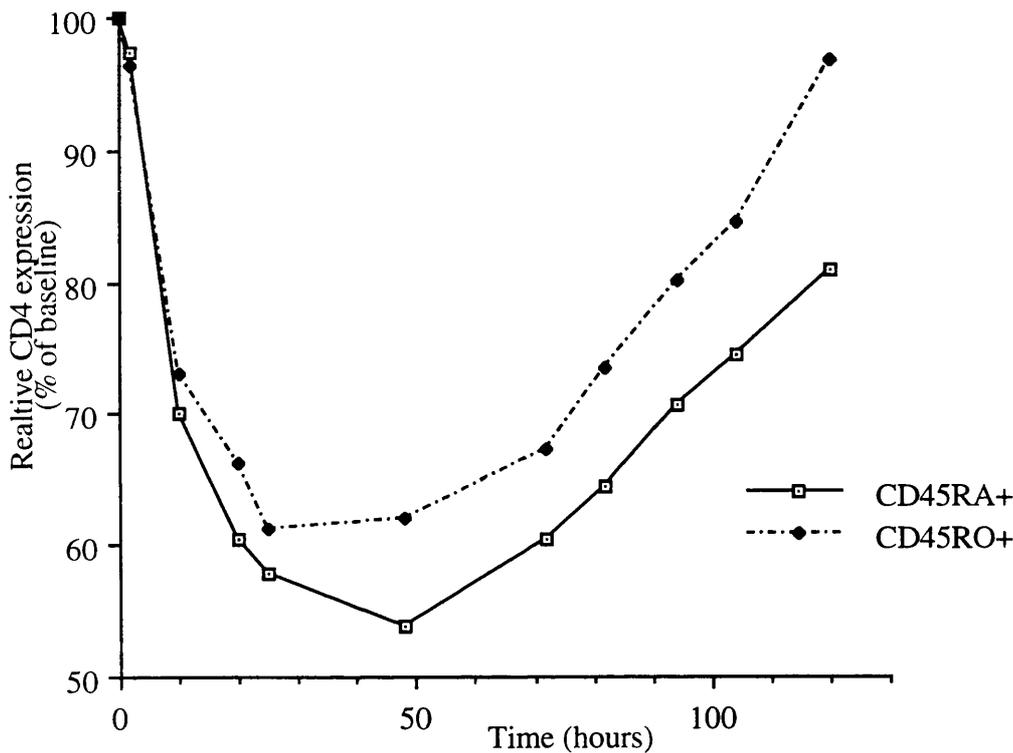


Figure 4.4 shows exposure to gp120 reduces CD4 expression over 24 hours, as has previously been noted in T cell lines<sup>1, 3</sup> and fresh lymphocytes<sup>5</sup>. Existing data<sup>83</sup> have shown that both CD45RA+ and RO+ populations down - regulate CD4 equally after exposure to un - cross - linked - anti CD4, although in these experiments CD4 expression was monitored for the first 50 minutes only. There is a subsequent slow re - expression of CD4 over the next two days, which has previously been shown to be due to *de novo* synthesis of CD4<sup>5</sup>. In four experiments CD45RO+ cells down - regulated CD4 less than CD45RA+ cells, with the former making a more rapid recovery over about 5 days.

In order to investigate the roles of different activation signals in the modulation of CD4 expression after exposure to gp120, cells were incubated with either tyrosine kinase (TK) inhibitor genistein (100 $\mu$ g/ml) or the PKC inhibitor H7 (50 $\mu$ M) for 1 hour prior to incubation with gp120.

Figure 4.5 CD4 Down - Regulation After gp120 And Genistein

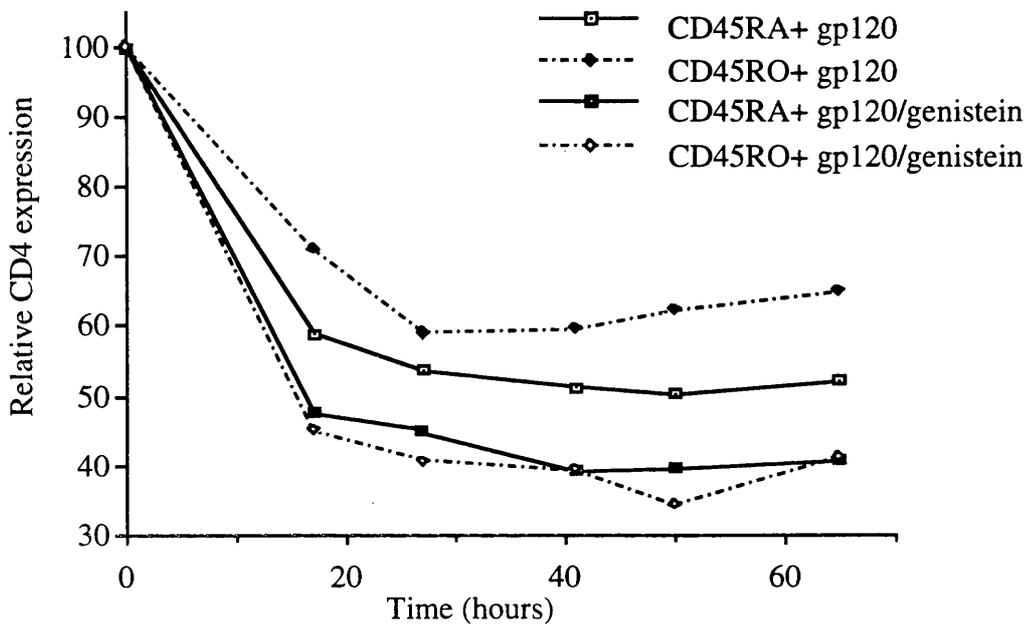
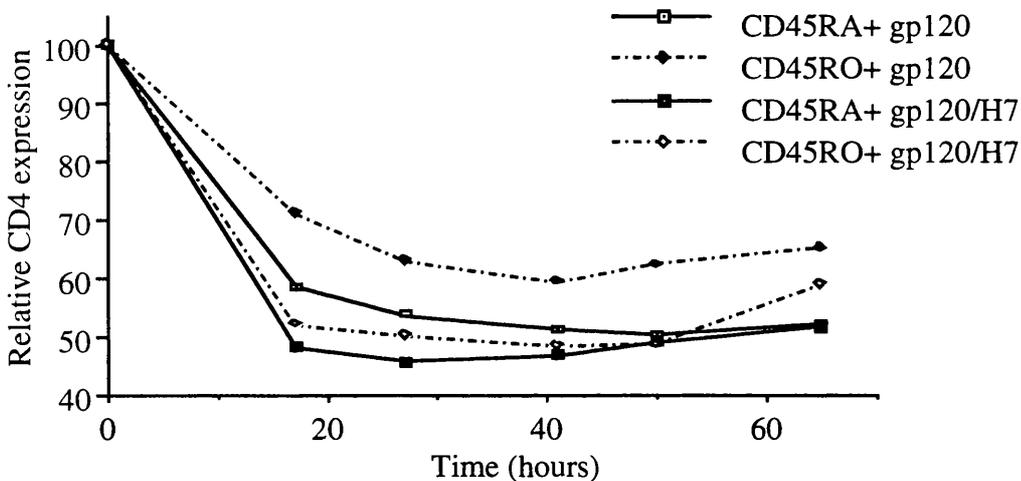


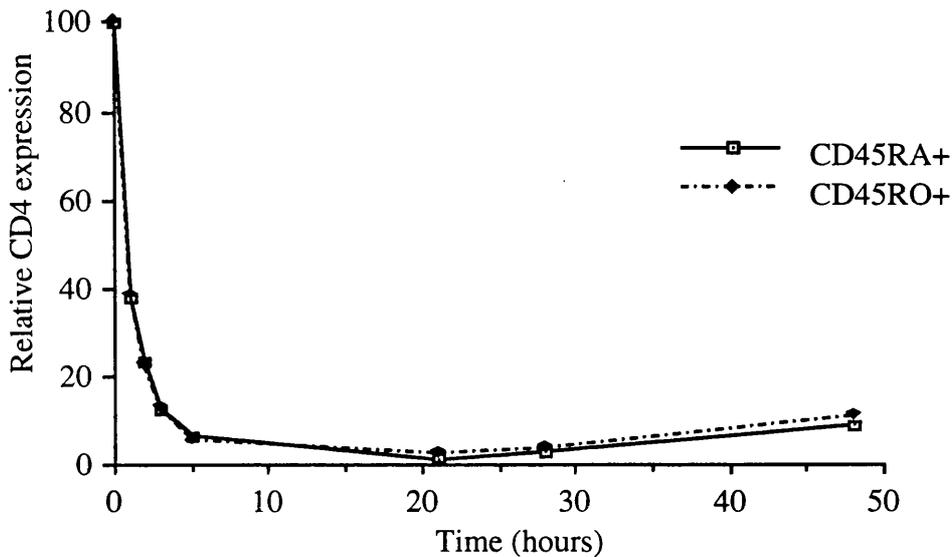
Figure 4.6 CD4 Down -Regulation After gp120 And H7.



It can be seen from figures 4.5 and 4.6 that inhibition of PKC and of tyrosine kinases particularly augment the down - regulation of CD4 upon ligation, as previously noted<sup>59</sup>.

Activation of PKC is known to completely down - regulate CD4, by dissociating it from p56<sup>Lck</sup><sup>8</sup>. To investigate the effects of PKC activation in the two populations, cells were incubated with phorbol - di - butyrate (PDBu; 1%) for 1 hour then washed extensively before monitoring CD4. The advantage of phorbol - di - butyrate is that it is water soluble and so the duration of PKC activation can be controlled by washing out.

Figure 4.7 CD4 Down - Regulation After Phorbol - di - butyrate.



Activation of PKC produced a more rapid and complete down - regulation of CD4 than gp120, with nearly all CD4 lost within 5 hours and affecting both populations equally. These confirm and extend previous data showing CD4 to be equally rapidly down - regulated on CD45RA+ and RO+ cells during the first 50 minutes of PKC activation<sup>83</sup>.

### 4.3 Discussion.

These data show that gp120 transmits signals in both CD45RA+ and CD45RO+ CD4+ cells, resulting in calcium influx, impairment of anti - CD3 mediated calcium influx and down - regulation of CD4. The calcium influx imparted by gp120 implies hydrolysis of inositol polyphosphates (although this could not be shown) and , more proximally, activation of tyrosine kinase(s). p56<sup>Lck</sup> is a likely candidate for this role, as discussed above. Basal p56<sup>Lck</sup> activity in resting CD45RA+ and CD45RO+ cells can be inferred to be the same since, both populations express similar amounts of CD4, and recent data<sup>83</sup> have shown similar amounts of p56<sup>Lck</sup> protein and p56<sup>Lck</sup> activity in both populations. There is no evidence that basal levels of PLC $\gamma$ 1 or PIP<sub>2</sub> are increased in CD45RO+ cells<sup>54</sup>. Likely explanations of the greater calcium influx in CD45RO+ cells are that p56<sup>Lck</sup> activity or phosphotyrosine association is increased preferentially after gp120 binding. This is consistent with data showing CD4 ligation by Class II molecules activates CD45RA+ cells in different ways from CD45RO+ cells<sup>61</sup>. Since CD45 regulates these p56<sup>Lck</sup> kinase activities after CD4 ligation<sup>62</sup>, it is likely that CD45 itself is responsible for this difference. On the other hand, ligation of CD3 has been shown to result in identical TK activity in CD45RA+ and RO+ cells<sup>63</sup>, suggesting that linkage of CD3 to proximal signalling mechanisms is the same in both populations of cells.

The extracellular differences in the CD45 isoforms may confer different linkages to CD4/p56<sup>Lck</sup> (see 1.1.6 and 1.2.2), resulting in differences in TK activity after CD4 ligation<sup>61</sup>. These differences in TK activity with different isoforms on CD45 are most readily seen when changes in adhesion molecule affinity are used as a measurement<sup>61, 64</sup> and may involve binding of CD45 to its (undefined) ligand<sup>65</sup>.

The enhanced calcium influx response to anti - CD3 in CD45RO+ cells is well known<sup>54-56</sup> but remains unexplained because only differences in more distal activation steps have been identified and ascribed to recent activation *in vivo*<sup>54</sup>. It remains possible that p56<sup>Lck</sup> is more effectively connected to CD3 when the CD45 isoform available is CD45RO.

CD3 signals were inhibited in both populations by prior treatment with gp120. Since gp120 binding does not down - regulate CD3<sup>1</sup>, this impairment of CD3 signal must occur through the T cell signalling mechanism. Gp120 has been shown to inhibit CD3 mediated stimulation without necessarily inducing a detectable calcium signal<sup>9, 57</sup>, probably by dissociating p56<sup>Lck</sup> from CD4 and the T cell signalling mechanism<sup>3, 66</sup>. Activation of p56<sup>Lck</sup> by gp120 also leads to phosphorylation of a different set of proteins than those induced by anti - CD4<sup>2</sup> and these may also hinder subsequent activation through CD3<sup>2</sup>.

The mechanism for CD4 down - regulation after gp120 binding is obscure. It does not involve phosphorylation of CD4 serine residues, as occurs after pharmacological activation of PKC, since this is much more rapid, almost complete and affects both populations equally (see figure 4.5). Furthermore, inhibition of PKC augments CD4 down - regulation after exposure to gp120 (see figure 4.6) and has no effect on anti - CD4 mediated CD4 down - regulation<sup>59</sup>.

CD4/p56<sup>Lck</sup> dissociation, p56<sup>Lck</sup> activation and CD4 down - regulation occur concurrently after gp120 binding<sup>3, 4</sup>. Dissociation of CD4 from p56<sup>Lck</sup> results in enhanced endocytosis of CD4 via clathrin coated pits<sup>67, 68</sup>, providing a more likely explanation of how CD4 is down - regulated after ligation. Possible explanations for how associated p56<sup>Lck</sup> prevents CD4 from entering the endocytosis pathway include p56<sup>Lck</sup> physically blocking entry into the clathrin coated pits, masking the CD4 endocytosis signal or by associating with cytoplasmic proteins, including actin (discussed in 68). Activation of the TK activity of p56<sup>Lck</sup> protects against CD4 down - regulation<sup>59</sup>.

The initial phase of down - regulation of CD4 after exposure to gp120 was more rapid in CD45RA+ than CD45RO+ cells (see figures 4.4 - 4.6) and the subsequent return towards normal CD4 expression over the next 5 days is earlier in CD45RO+ cells. Previous data have only monitored the early phase of CD4 down - regulation, which was similar in both CD45RA+ and RO+ populations<sup>83</sup> after PKC activation or CD4 ligation, and have been used to support the argument that p56<sup>Lck</sup> activity is alike in these populations<sup>83</sup>. The data presented here suggest p56<sup>Lck</sup> tyrosine kinase activity protects against CD4 down - regulation and that it may be that increased p56<sup>Lck</sup> activity (phosphotyrosine binding ability or TK activity) in CD45RO+ cells inhibits

CD4 down - regulation after gp120 binding. Whether these differences are actually direct consequences of the CD45 isoform expressed on the cell, or another co - factor molecule is not clear.

Other factors may also down - regulate CD4 expression in lymphocytes infected with HIV; expression of the gp160 ligates CD4 in the endoplasmic reticulum, rendering p56<sup>Lck</sup> inaccessible to the cell surface<sup>69</sup> and signalling mechanism. The HIV regulatory peptide nef down - regulates CD4 expression<sup>70, 71</sup>, possibly by inhibiting the cellular transcriptional machinery<sup>72</sup>. CD4 down - regulation may contribute to the signalling defect in these cells<sup>73</sup> and also to super - infection protection, the process used by retroviruses to ensure that infected cells do not become re - infected. Multiple rounds of HIV infection cause cytopathic effects<sup>74-76</sup>, due to accumulation of un - integrated proviral DNA<sup>77</sup>. *In vivo* super - infection is rare<sup>78</sup> and not a prominent cause of cell depletion in AIDS pathogenesis emphasising the importance of CD4 down - regulation and superinfection protection..

The abnormal signals induced by soluble gp120 binding inhibit CD4+ T cell responses to anti - CD3. When cells respond to antigen abnormal signalling is compounded by inhibition of Class II binding by gp120<sup>9, 18</sup>. Hence CD4+ cells responding to antigen fail to proliferate as a result of gp120 induced abnormalities<sup>1</sup>. This may contribute to abnormalities seen early in HIV infection at a time when CD4+ T cell counts remain normal<sup>79-81</sup>.

Candidate HIV vaccines based on gp120 face a number of theoretical obstacles. Of particular concern is that gp120 may induce abnormal signalling, even in healthy vaccinees, although this has not been seen, even in volunteers receiving up to 1g of gp120<sup>84</sup>. The enormous variability of gp120 means that gp120 vaccines are likely to confer immunity only against specific strains of HIV. To date, 5 gp120 vaccinees have subsequently become infected with HIV. Two of these individuals appear to have had very rapid disease progression following infection, reiterating the uncertainty as to whether anti - gp120 antibodies are protective or whether they are involved in pathogenesis.

In conclusion, CD4+ cells transmit signals after gp120 ligation, more prominent in CD45RO+ cells. Although these may contribute to immunosuppression the concomitant p56<sup>Lck</sup> activation may make CD45RO+ cells less vulnerable to the associated CD4 down - regulation. More stable CD4 expression and enhanced signalling may contribute to the greater ability of CD45RO+ cells to form syncytia, the subject of the next chapter.

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## Chapter 5. HIV Syncytium Formation In CD4+ CD45RA+ And CD45RO+ T Cells.

Syncytium formation (SF) is a major means of cell to cell spread of HIV and causes cytopathology. SF is easily detected *in vitro* and has thus been used as a surrogate for virus/cells fusion. Although syncytia are rarely seen in *ex vivo* samples, the presence of syncytia inducing HIV strains is linked to disease progression<sup>1-5</sup> and has led to the idea that SF occurs *in vivo* and contributes to pathogenesis.

### 5.1 Virus Fusion And Syncytium Formation.

Fusion of cell free virus to cell membranes (“fusion”) and SF between infected and uninfected cells are obligate steps in the life cycles of many viruses. In the influenza virus model an envelope glycoprotein is cleaved to a cell binding fragment and a hydrophobic fragment, which penetrates the cell membrane.

Although the CD4 molecule is endocytosed, via clathrin coated pits<sup>6</sup>, cell free HIV does not require endocytosis after binding to CD4<sup>7</sup> and so appears to fuse with the cell membrane at the cell surface. Although fusion and SF both occur at the cell surface, they do not appear to be identical processes; for example, regions in CD4 can be required for both processes or just one. This chapter focuses on the mechanism of SF.

Minimal requirements for SF include HIV gp120 and gp41, which, as in the influenza model, dissociate in order to function. To become infected with HIV a T cell must express correctly configured CD4 and an unidentified molecule expressed most frequently on human cells<sup>8</sup>. Experiments using heterokaryons have shown that this molecule requires several chromosomes and so is probably the product of a series of enzymes; the most likely candidate is that this molecule is a cerebroside<sup>9</sup>. Adhesion

molecules, intact signalling pathways and, possibly, cell surface proteases contribute to the SF process.

### 5.1.1 CD4 And Syncytium Formation.

The well defined role of the CDR2 region in gp120 binding has been discussed in Chapter 3. Antibodies against the CDR3 region of the V1 domain of CD4 either inhibit SF without affecting gp120 or virus binding<sup>10</sup>, or alternatively impede SF without affecting cell free HIV infection<sup>11</sup>. These data suggest CDR3 has a different role from that of the physically separated CDR2 region<sup>12,13</sup> (see figure 1.2). Chimpanzees, which show viraemia but no syncytia after infection with HIV, possess CD4 molecules which bind gp120 with the same kinetics as the human molecule, but do not support syncytia even when transfected in to human cells. Since the only difference between CD4 from the two species is the replacement of Glu with Gly at residue 87 of CDR3, this residue is likely to be involved in SF<sup>14</sup>.

There are a number of possible explanations for CDR3 (residue 87 in particular) being important in SF but not in gp120 binding. For example CDR3 is required in the transition from an unstable CD4/gp120 structure, known as an “encounter complex”, to a stable complex<sup>15, 16</sup> held together by stronger Van der Waals forces (see section 3.3.). CDR3 may also be involved in the dimerisation of CD4<sup>17</sup> and the CD4 ligands for MHC Class II<sup>18</sup> and gp120<sup>19</sup> both exist as oligomers. Although monomeric CD4 binds gp120, SF may only be able to progress when CD4 is dimerised. CDR3 may also be involved in the cleavage of gp160, releasing gp120 and exposing gp41 to facilitate subsequent SF<sup>20</sup>, although this finding awaits confirmation. Finally, the CDR3 region may be necessary for the transmission of signals<sup>21</sup>, by virtue of its role in dimerisation or by *cis* association with other T cell surface molecules<sup>21</sup>. CD4 cell signalling in turn is required for SF<sup>18</sup> (see below).

In other studies however, mutations in the CDR3 residue 87 were found to have no effects on SF<sup>22</sup>. The authors suggest that the negatively charged CDR3 non-specifically interacts with the positively charged V3 loop of gp120 to promote release of this molecule from gp41. Negatively charged sulphated polysaccharides also inhibit SF and promote gp120 release.

Experiments with rat/human CD4 chimeras have shown that rat CDR3 domains in human CD4 are enough to confer susceptibility to HIV infection and SF<sup>9, 23</sup>. These data have been used to argue that CDR3 is not important in these processes, but the possibility remains that CDR3 domains from some species can substitute for human CDR3.

Antibodies against the V3 domain of CD4 do not block gp120 binding but block both viral fusion and SF<sup>24</sup>. These antibodies may alter the conformation of a putative hinge region<sup>12, 13</sup> between V2 and V3, thus preventing flexion of the CD4 molecule and interaction of the target cell surface with virus. Gp120 binding partially inhibits binding of these molecules to the V3 region, suggesting that gp120 binding changes the

conformation of CD4. Two other antibodies against the V3/V4 domains inhibit fusion with no effect on binding of gp120 or SF<sup>25</sup>. These antibodies impair anti - CD3 mediated calcium signals and so may impede viral entry by affecting signal transduction.

### 5.1.2 Activation Requirements For Syncytium Formation.

HIV infection occurs preferentially in activated T cells<sup>26</sup>. The protein kinase C inhibitor H7 causes cell free HIV to accumulate on the T cell surface after binding<sup>27</sup>. Although this has not been seen in all experiments<sup>28</sup>, it implies that cell free HIV infection also requires an activation signal through PKC. The findings that SF requires calcium ions, is inhibited by sodium azide<sup>29</sup>, and is temperature dependant<sup>30</sup> not only favour a role for intracellular signalling, but are also requirements for integrin binding.

A series of chemically mutated subclones of a parent T cell line showed variable SF despite similar expression of LFA - 1, CD4 epitopes and gp120 binding.<sup>31</sup> Clones with impaired SF had reduced gene transcription<sup>32</sup> implying that poor signal transduction correlates with impaired SF, since protein (and nucleic acid) synthesis is not required for SF<sup>33</sup>.

In monocytes transfection of p56<sup>Lck</sup> facilitates SF in a fashion dependent on TK activity<sup>34</sup>. Similarly, cytoplasmic tail - less CD4 is less efficient at supporting SF than intact CD4 in lymphoid cells<sup>35</sup>. Glycosylphosphatidylinositol anchored CD4 also supports cell free infection less well than intact CD4<sup>36</sup> although these experiments were carried out in HeLa cells, suggesting that absence of p56<sup>Lck</sup> association did not account for the difference.

In summary, activation signals appear to enhance both SF and viral fusion. Signalling through CD4, p56<sup>Lck</sup> and subsequently PKC have roles in this process.

Activation may increase SF by altering cell surface charge or membrane fluidity, although increasing the affinity of adhesion molecules, through inside out signalling, seems the most likely mechanism.

### 5.1.3 Inter - Cellular Adhesion And Syncytium Formation.

A number of molecules have some effects on SF, including MHC Class II (also in viral fusion), selectins<sup>37, 38</sup>, MHC Class I<sup>39</sup> and LFA - 3<sup>38</sup>, but the most potent affects on SF are mediated by the LFA - 1/ICAM - 1 ligand pair. In studies using cells from individuals with the leukocyte adhesion deficiency syndrome, in which LFA - 1 expression is defective, or monoclonal antibodies against LFA - 1 and ICAM - 1, the involvement of these molecules in HIV mediated SF was shown<sup>40-42</sup>. LFA - 1/ICAM - 1 binding enhances SF, but has never been shown to be an absolute requirement.

One putative role for LFA - 1 was that it ligated gp41, the HIV fusion glycoprotein; subsequently this was refuted<sup>43</sup> and LFA - 1 on the un - infected syncytium partner and ICAM - 1 on the infected cell were shown to be the required

ligands<sup>43, 80</sup>. Anti - ICAM also inhibits cell free HIV infection<sup>80</sup>, suggesting ICAM molecules may be present on the virion surface. This binding appears not to be important at the very early stage of single cell fusion, but later, when giant cells are forming<sup>44</sup>. Refuting this idea is the finding that epitopes on adhesion molecules involved in inhibiting HIV infection are not the same as those that block homotypic adhesion, suggesting that the role of these molecules is more complex than simply promoting intercellular adhesion<sup>38</sup>.

Cell free HIV is associated with expression of a number of host cell proteins, notably MHC Class I and II, CD11 $\alpha$  and CD18<sup>45</sup>. There appears to be an active process of recruitment of these molecules on to the virus surface, unrelated to host cell expression density and excluding CD4 and CD45<sup>45</sup>. Hence expression of LFA - 1 on un - infected cells may be expected to have an impact on cell free HIV; experimental data show LFA - 1 antibodies can inhibit cell free infection of lymphocytes<sup>38, 41, 46</sup> although this is not always as striking as with SF.

The role of integrins in SF may explain the requirement for signalling, since T cell activation signals can up - regulate adhesion<sup>47</sup>; inhibition of signalling also inhibits integrin binding.

The T cell surface protease, CD26, has been proposed as a major surface co factor for cell free virus infection<sup>48</sup>. The proposed mechanism is that this protease degrades gp120 at the V3 loop. CD26 is up regulated on CD45RO+ T cells and CD26+ cells have been noted to diminish in number in HIV infection<sup>49, 50</sup>. However, subsequent studies have failed to show any effects of CD26 on fusion or SF. An additional study has found HIV that proviral carriage was greater in CD26- cells and suggested that CD26 protected against infection<sup>51</sup>.

In summary, SF depends on gp120 binding to the CD4 CDR2 domain, the CDR3 "fusion domain" and the V3/V4 hinge. CD45RA+ or RO+ CD4+ T cells both express similar amounts of these three regions and bind gp120 equally well (see chapter 3). LFA - 1 is up - regulated on CD45RO+ CD4+ cells<sup>52, 53</sup> thus promoting adhesion during SF. However, there may also be qualitative differences in adhesion molecules on the two populations of cells; LFA - 1 increases its affinity after PKC activation whilst other molecules become more adhesive after TK is activated<sup>54, 55</sup>. These signalling mechanisms may be modulated differently by specific isoforms of CD45 and may favour adhesiveness in CD45RO+ cells<sup>54, 56</sup>. As shown in chapter 3, CD45RO+ cells recovered CD4 expression after gp120 mediated downregulation; ability to continue expressing CD4 after exposure to gp120 may also facilitate SF.

Hence SF may occur preferentially in CD45RO+ cells for a number of reasons, some dependent on CD45 itself and some on associated molecules. To test this hypothesis and explore the relative influence of different factors, separated CD45RA+ and RO+ cells were tested for their ability to form syncytia.

## 5.2 Results.

Since CD45RA+ and CD45RO+ cells are known to differ in their level of activation and integrin expression, it was conceivable that SF may proceed at different rates in these populations. The experiments in this chapter aim to discern whether this is the case.

### 5.2.1 Syncytium Formation In CD45RA+ and CD45RO+ CD4+ Lymphocytes.

H9 cells were infected with HTLVIIIIB and then maintained for at least 50 passages. These chronically infected cells (called H9IIIB) were shown to have little active HIV replication (supernatant p24 less than 20ng/ml, supernatant viral titre less than 100 infectious units / ml). CD4+ CD45RA+ and CD45RO+ cells ( $5 \times 10^5$ ) were mixed with  $10^5$  H9IIIB cells and incubated for up to 3 days. Syncytia, defined as cells with cytoplasmic blebbing and multinucleate giant cells, from 4 high power fields were counted by a “blinded” observer (fig 5.1.) The average number of syncytia per high power field and standard deviation for each experiment were then calculated.

Figure 5.1. Syncytia Formation 48 Hours After Mixing Lymphocytes And H9IIIB.

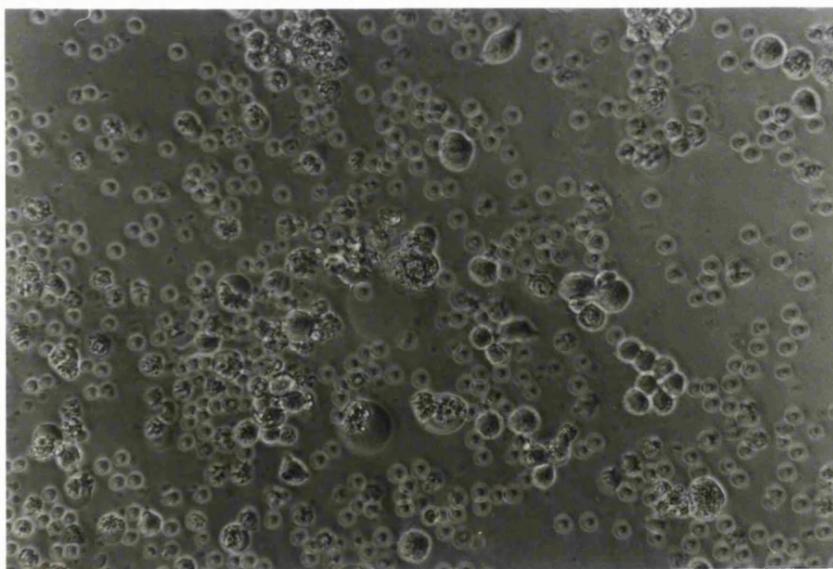


Figure 5.2. Syncytia In CD4+ CD45RA+ And CD45RO+ Cells After Mixing With H9IIIB

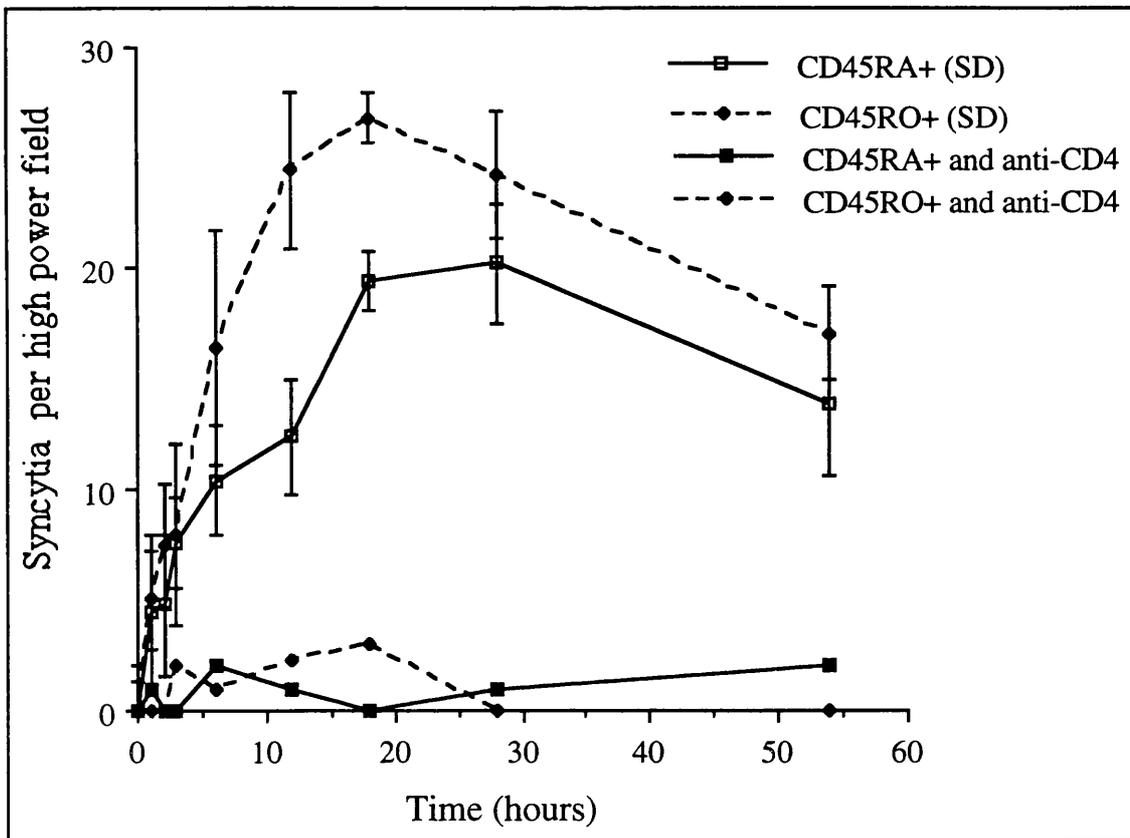
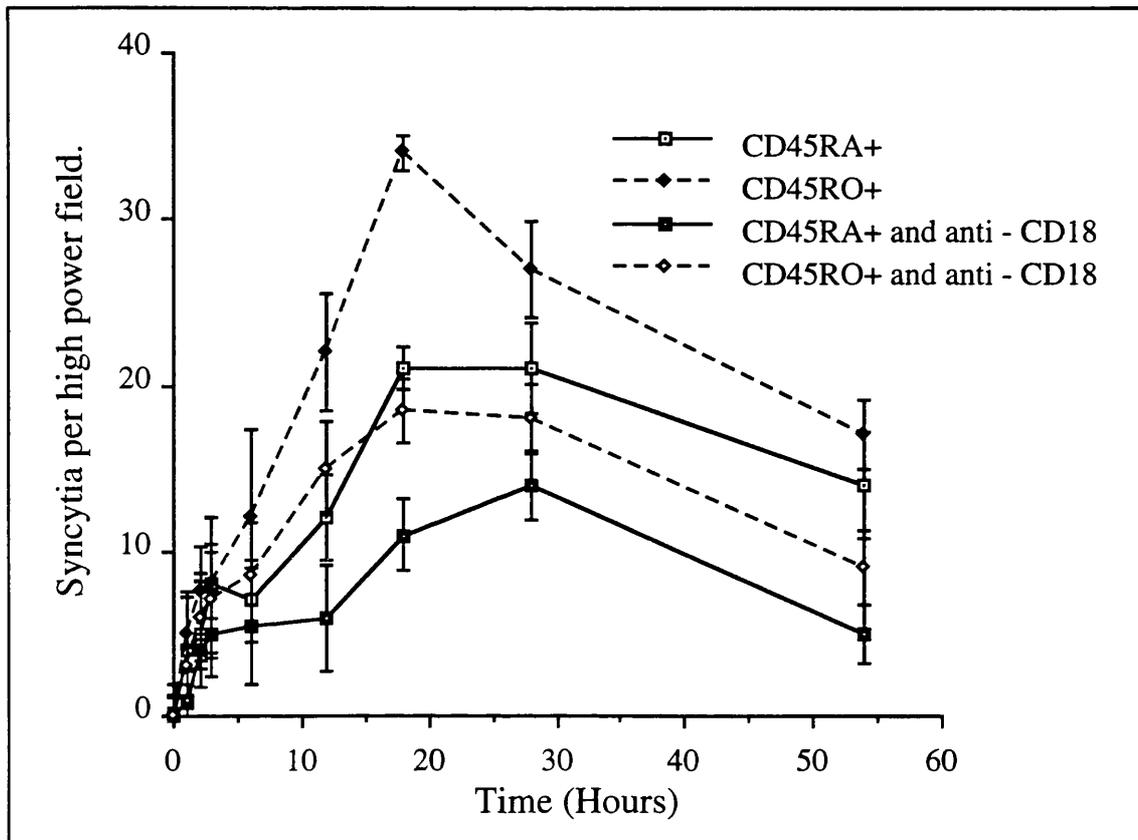


Figure 5.2 shows data from a typical experiment; the error bars show the standard deviation for the 4 high power fields in which syncytia were counted.

It can be seen that treating separated fresh lymphocytes with anti - CD4 (QS4120) and washing, before mixing with H9IIIIB almost completely inhibited SF, suggesting that in the absence of anti - CD4 the majority of SF are occurring between lymphocytes and H9IIIIB and not just within the H9IIIIB population. Furthermore, rather more syncytia are present in wells containing CD45RO+ cells than in those containing CD45RA+ cells. There was no difference in the morphology of syncytia produced by the two populations, suggesting that CD45RA+ do not simply produce fewer large syncytia, but are less efficient at SF than CD45RO+ cells. The kinetics of SF are favoured in CD45RO+ cells; the number of SF declines when all H9IIIIB are involved in syncytia and have undergone lysis.

SF is augmented by the expression of the adhesion molecule pair LFA - 1<sup>40-42, 46</sup>, which is increased on CD45RO+ cells<sup>52, 53</sup>. Immunofluorescent staining of CD45RA+ and RO+ cells used in these experiments showed both populations to be positive for CD18, although median fluorescence intensity was higher in the CD45RO+ populations (RA+ 49.1 fluorescent units, RO+ 82.8 units). To study the impact of these molecules on SF the anti - LFA - 1 $\beta$  (CD18 ) antibody MHM23<sup>57</sup> was added at 5 $\mu$ g/ml at the same time as mixing syncytium forming partners.

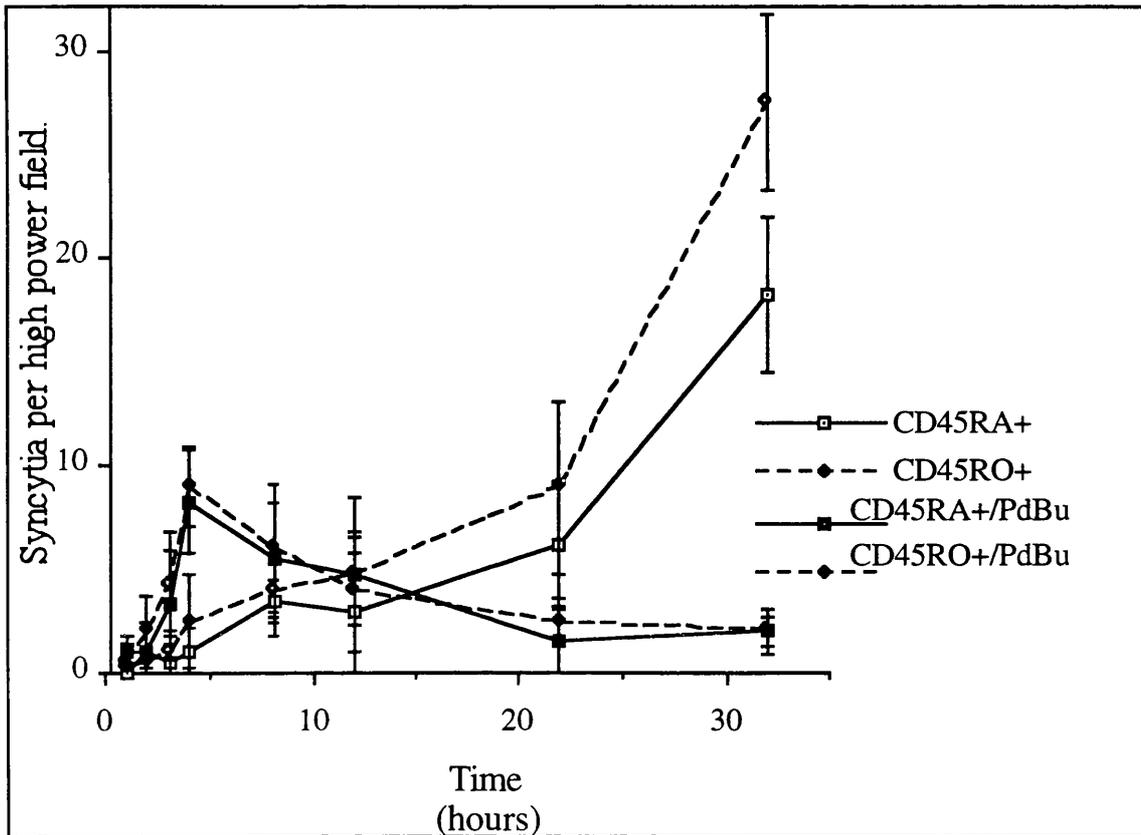
Figure 5.3. Effect Of Anti - LFA - 1 $\beta$  On Syncytium Formation.



It can be seen in figure 5.3 that addition of anti - LFA - 1 $\beta$  reduced the number of syncytia seen in both populations, however there were still more syncytia in the CD45RO+ population even when LFA - 1/ICAM - 1 was not functional. These data suggest that this pair of molecules contribute to SF but do not account for all the difference between the two populations.

Adhesion molecule (including LFA - 1/ICAM - 1) interactions are promoted, via “inside - out” signalling by PKC activation<sup>55</sup> and, in some cases, TK signalling<sup>54</sup>. PKC activation has been noted to up - regulate SF at 4 hours or less<sup>55,58</sup>, but decrease SF at 20 hours<sup>59</sup>. To assess the impact of PKC activation on SF in CD45RA+ and RO+ populations, cells were incubated with PdBu (1%) for 1 hour, washed extensively and then mixed with H9IIB. PdBu, rather than PMA, was used because it produces transient activation of PKC.

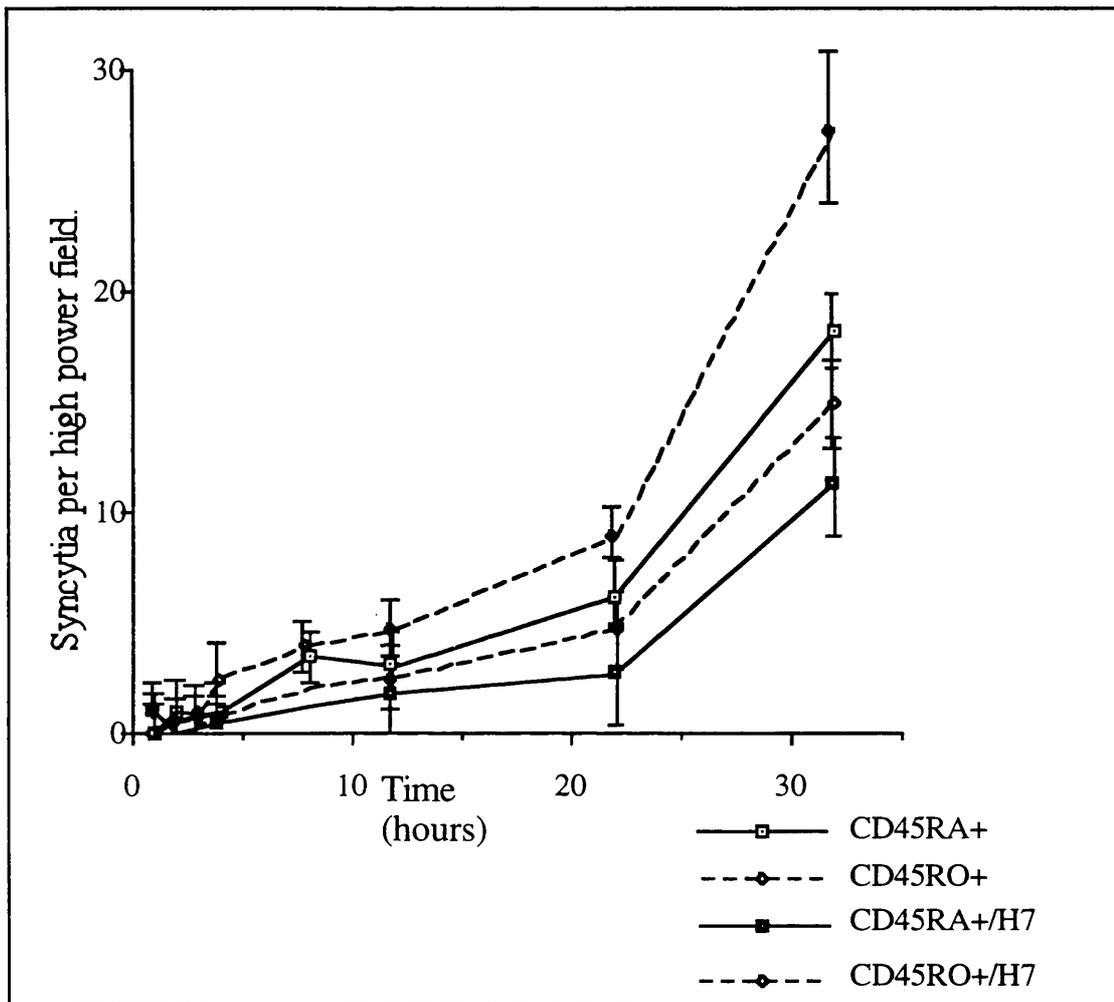
Figure 5.4. Effect Of PdBu On Syncytium Formation.



As can be seen in figure 5.4, PKC activation resulted in enhanced early SF, equal in both populations and peaking at 4 hours. Subsequently, there was a decline in SF, probably because of the concomitant down - regulation of CD4 seen when PKC is activated<sup>60</sup> (see chapter 4). These changes were seen in 3 experiments and, most importantly, affected both populations equally.

If greater baseline PKC activity accounts for the difference between CD45RA+ and RO+ populations, inhibition of PKC would be expected to equalise SF. The effect of inhibiting PKC was studied by pre - treating cells with H7 50 $\mu$ M (figure 5.5). PKC inhibition resulted in fewer syncytia in both populations, with CD45RO+ cells producing marginally more syncytia, in a manner resembling blocking of CD18 (figure 5.3). Hence, in untreated cells the higher basal PKC activity in CD45RO+ cells<sup>61, 62</sup> may facilitate SF.

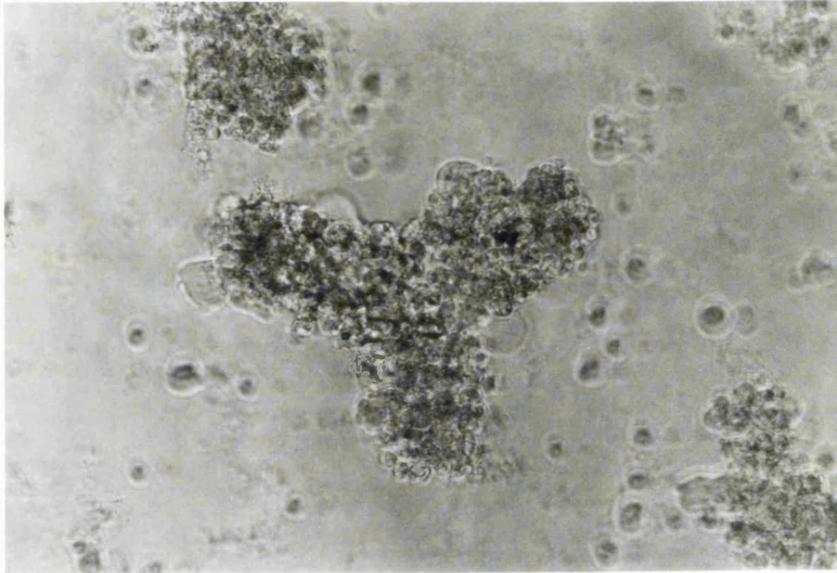
Figure 5.5 Effect Of H7 On Syncytium Formation.



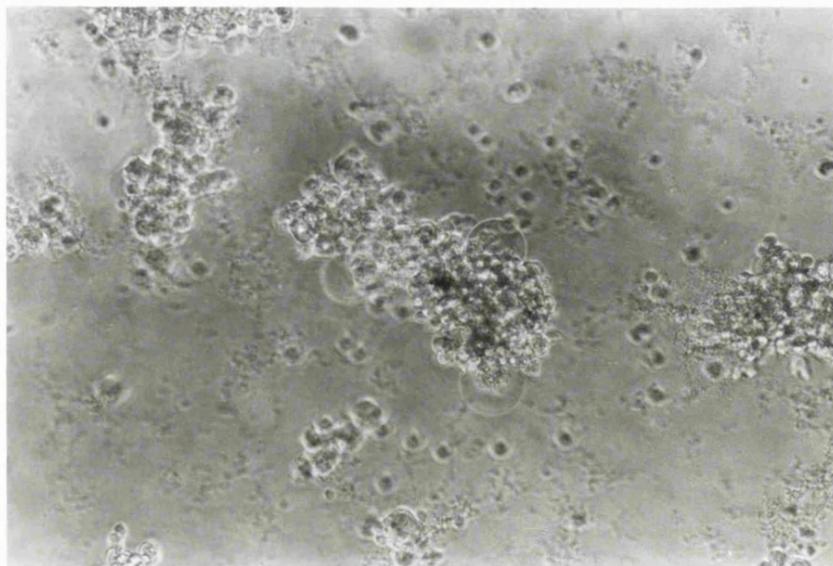
Binding of gp120 to CD4 results in signalling through TK (see chapter 4), which may affect adhesion and hence SF. To up - regulate TK signals in CD45RA+ and RO+ cells CD45 was cross linked, a procedure which can activate TK<sup>54</sup> and modify signalling through CD4<sup>63</sup>, resulting in enhanced SF<sup>54</sup>. Separated CD45RA+ and RO+ cells were incubated with saturating quantities of anti CD45RA (SN130) or anti - CD45RO (UCHL1), respectively, washed and then incubated with rabbit anti - mouse immunoglobulin (RAM) and washed again prior to mixing with H9IIIIB. Cells incubated with RAM alone were used as a control. In both populations of cells CD45 cross linking led to very rapid clustering and SF, compared to cells incubated with RAM (see figure 5.6). By two hours all cells were clustered, by four hours no normal lymphocytes were visible and by 18 hours all that remained was cellular debris. This process affected both populations equally.

Figure 5.6. Effect Of CD45 Cross Linking On Syncytium Formation In CD45RA+ (A) And CD45RO+ (B) Cells, 2 Hours After Mixing With H9IIIB Cells.

A

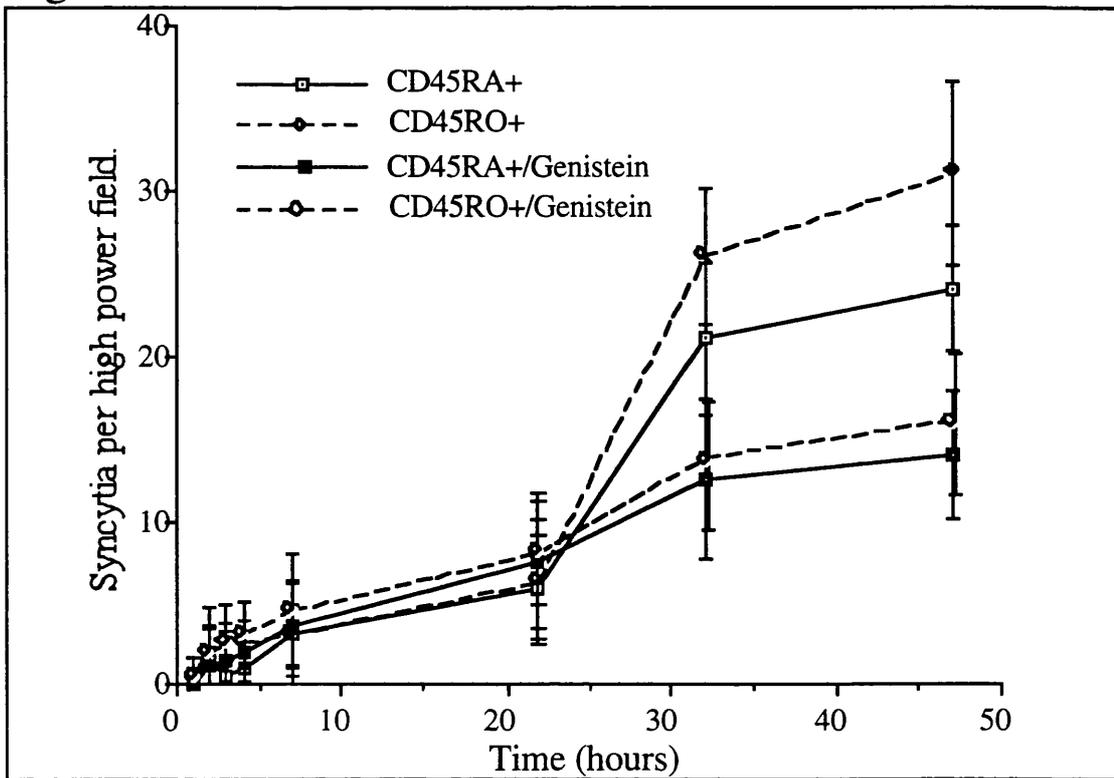


B



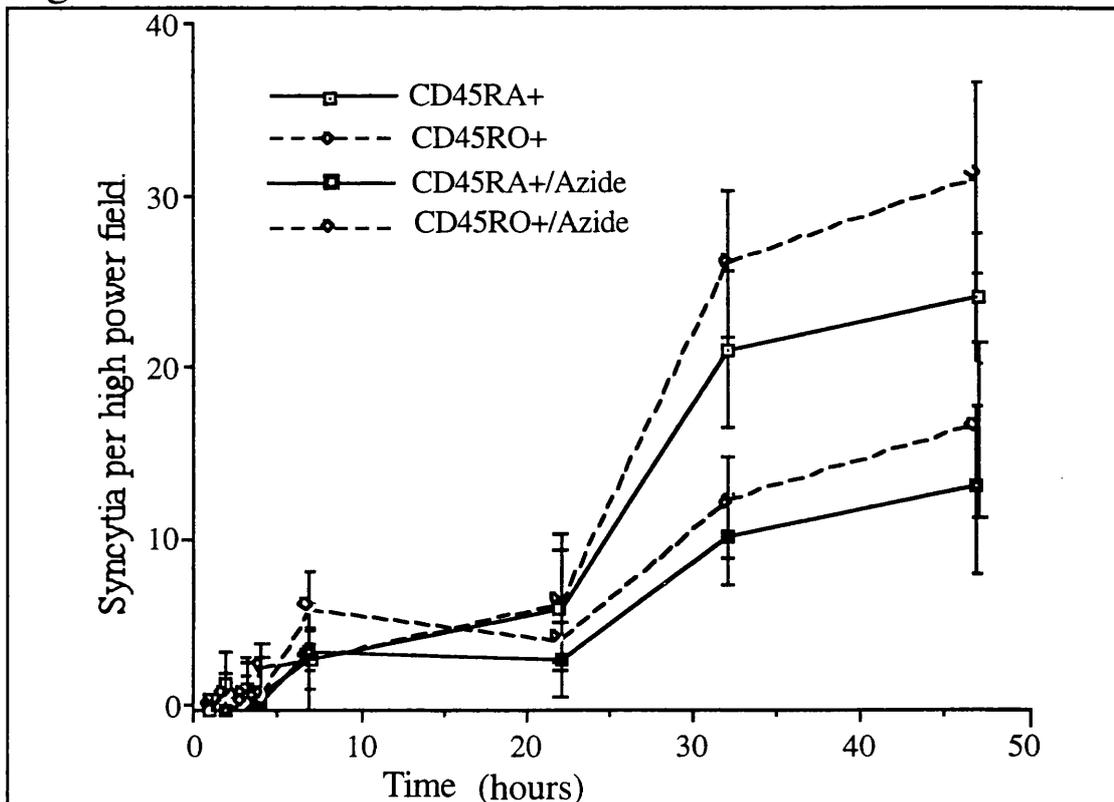
If cross - linking of CD45 increases SF by activating TK, inhibition of TK would be expected to have inhibitory effects on SF. To study the effect of TK on SF, separated lymphocytes were incubated for 1 hour with genistein (100 $\mu$ g/ml) prior to mixing with H9IIIB. Genistein inhibits TK activation on CD4 binding<sup>64</sup>. As seen in figure 5.7 inhibition of TK resulted in fewer syncytia being formed, in the later part of culture particularly. Notably, SF in CD45RO+ and CD45RA+ cells was comparable when TK was inhibited. The inhibition of SF associated with genistein could be either because TK is needed to up - regulate adhesion or inhibits down - regulation of CD4 after CD4/gp120 binding (see chapter 4).

Figure 5.7. Effect Of Genistein On SF.



To confirm that energy requiring processes, such as increased integrin affinity<sup>60</sup>,<sup>65</sup>, are involved in SF, lymphocytes were pre - treated with sodium azide (0.4%) for 1 hour, washed and mixed with H9IIB. Inhibition of metabolism decreased SF, as noted previously<sup>29</sup>, although CD45RO+ maintained a slight advantage (figure 5.8).

Figure 5.8. Effect Of Sodium Azide On SF.

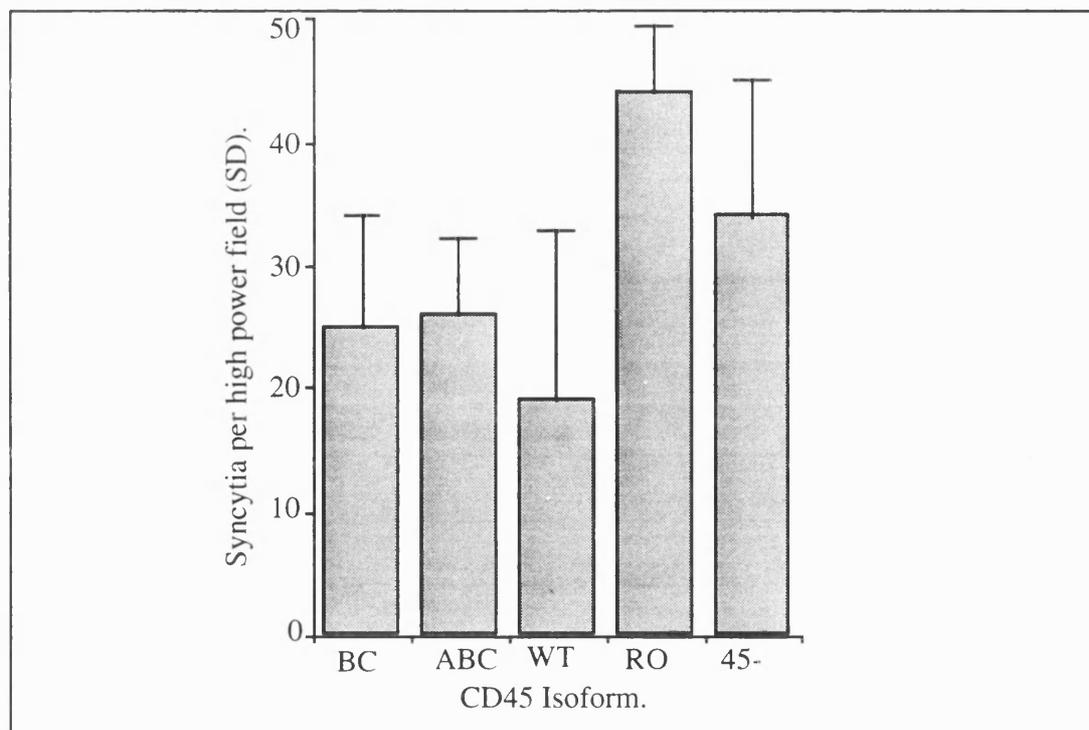


### 5.2.2. Syncytium Formation In HPBALL Cells Expressing Different Isoforms Of CD45.

The differences in SF between CD45RA+ and RO+ CD4+ lymphocytes may be attributed to the isoforms of CD45 itself or to other differences between the populations. To study the influence of CD45 on SF more directly, HPBALL cells were used. HPBALL is a CD4+ CD8+ T cell leukaemia line, which can spontaneously lose CD45 expression<sup>66</sup>. The HPBALL parent lines expresses mainly CD45RB, with much lower amounts of CD45RA and CD45RO<sup>67</sup>. Spontaneous mutants, with undetectable CD45 were isolated by sorting<sup>68</sup> by L. Goff. These HPBALLCD45- cells contain CD45 DNA, but no CD45 mRNA or surface protein (L. Goff, personal communication). HPBALLCD45- were then stably transfected with genes for CD45RO, CD45RBC and CD45RABC, all of which were fully expressed. These HPBALL lines express similar amounts of CD4, but variable amounts of LFA - 1; for example the wild type variant expressed more LFA 1 than the CD45- cells (L. Goff, personal communication).

Cells expressing the wild type (B), ABC, and BC isoforms of CD45 produced rather fewer syncytia (at 48 hours, time of peak production) than cells expressing CD45RO or no CD45 (Figure 5.9). The observation was also made that CD45RO and CD45- cells produced cell aggregates, when cultured alone, whilst cells expressing the higher molecular weight isoforms produced free cells. Hence the high molecular weight isoforms inhibit both homotypic aggregation and SF.

Figure 5.9. Syncytium Formation In HPBALL Cells.



## Cell Free Infection Of HPBALL Cells Expressing Or Lacking CD45.

To assess whether CD45 expression also affected cell free infection of T cells, HPBALL cells either lacking or expressing wild type CD45 were infected at an MOI of 0.005 with 3 strains of HIV - 1 (LAV<sup>69</sup>, unpassaged, H9III B - LAV passed through the T cell line H9 and S1D6, LAV passed through the monocyte line Monomac 6<sup>70</sup>). Cells were examined at intervals for syncytium formation and aliquots of supernatant were taken for p24 estimation (ELISA - Dupont).

**Table 5.1. Syncytium Formation And p24 Production In HPBALL Cells After Infection With Cell Free HIV Strains.**

	LAV				H9III B				S1D6			
	Wild Type		CD45-		Wild Type		CD45-		Wild Type		CD45-	
	p24*	SF‡	p24	SF	p24	SF	p24	SF	p24	SF	p24	SF
3days	0	0.5	0	0	0	0	0	0	0	0	0	0
4days	0	1	0	3	0	0	0	0	0	1	0.3	2
5days	0	1	0	5	0	0	0.13	1	0.12	1	1.1	2

\*ng/ml

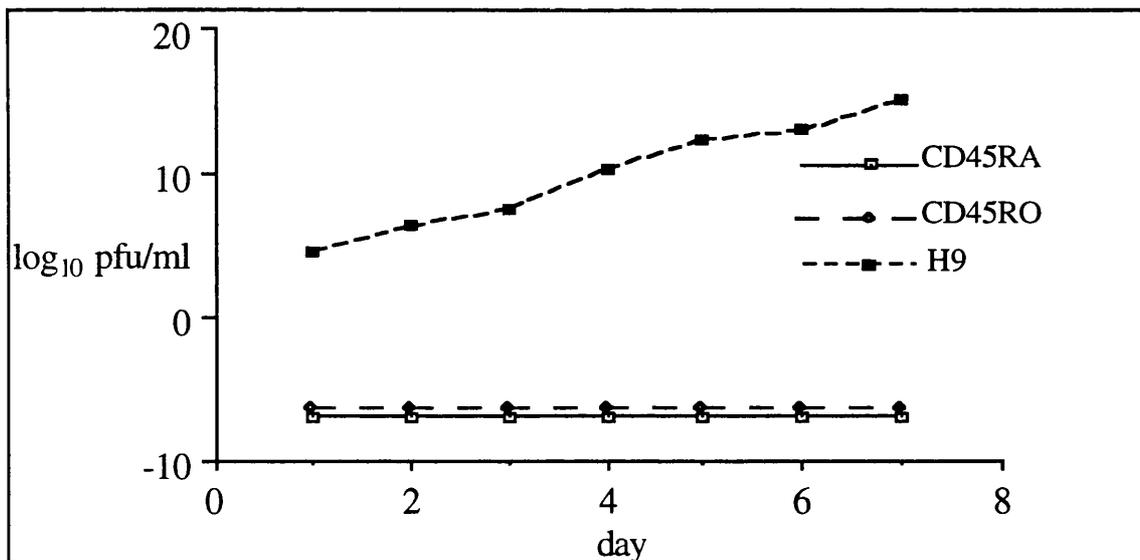
‡Syncytia Per High Power Field

The data in Table 5.1 show both SF and p24 production proceeded more rapidly in CD45- cells. With cell free infection it is less easy to attribute enhanced p24 production to more effective fusion in the CD45- cells, since it is known that in other CD45- HPBALL lines, NFkB is constitutively active and hence HIV protein transcription is up - regulated<sup>71</sup>. However, SF is increased in this population after cell free HIV infection, providing corroborative evidence for enhanced virus/cell fusion. An interesting feature of these data is that SF and p24 production did not always parallel one another, as has been noted previously<sup>59</sup>.

## Yellow Fever Virus Replication In CD45RA+ and CD45RO+ CD4+ Lymphocytes.

To assess whether increased infection and replication in CD45RO+ cells is a general feature of viruses, CD45RA+ and RO+ cells, along with the T cell line H9 were infected with yellow fever virus. Yellow fever virus was chosen because it is an RNA Flavivirus with no requirement for reverse transcription. After infection, extensive washing and culture, supernatant was taken at intervals and titrated to determine the level of replication.

Figure 5.10. Yellow fever virus replication in CD45RA+ and RO+ T cells and H9 cells.



The receptor and transcription factors for yellow fever virus are not characterised, however these data confirm that T cell lines are permissive for this virus. Unlike HIV, yellow fever virus does not replicate in either replicate CD45RA+ and RO+ T cells. CD45RO+ cells are thus not specially susceptible to all viruses and specific characteristics of HIV and its replicative cycle hence account for its enhanced ability to grow in CD45RO+ cells.

### 5.3. Discussion.

The data in this chapter show that in peripheral blood CD4+ lymphocytes the CD45RO+ population are modestly more efficient at SF than the CD45RA+ population. In the HPBALL T lymphocyte line expression of high molecular weight CD45 isoforms appears to impair SF and cell free infection, irrespective of LFA - 1 expression. Hence high molecular weight CD45 isoform expression is correlated, particularly CD45RA in peripheral blood lymphocytes, with inefficient SF.

Three mechanisms could contribute to the effects of CD45 on SF. Firstly the relatively high expression, intense glycosylation and size of CD45 means it may contribute to net repulsive forces between cells<sup>72</sup> (discussed in <sup>47</sup>). Since CD45 associates with CD4 and LFA - 1<sup>73</sup>, CD45 may cap at the interface between cells and physically hinder SF; this process may be prevented by cross linking or deletion of CD45, in the case of HPBALL. The lower molecular weight CD45RO isoform may be less able to form such an obstruction and hence SF is more efficient in CD45RO+ cells.

Secondly, activation of T cells could result in non - specific changes, such as surface charge and fluidity, which makes them more permissive to fusion with cells or viruses. This appears not to be the case with yellow fever virus, which infects CD45RA+ and CD45RO+ equally poorly, although it is possible that CD45RO+ T cells

do not cross an undefined threshold for yellow fever virus fusion. Con - focal microscopy could be used to confirm whether CD45 molecules can impose a physical block to SF.

The third possibility is that adhesion molecules are regulated differently on CD45RA+ and CD45RO+ cells. Expression of integrins is greater on CD45RO+ cells<sup>52, 53</sup> and so SF would be expected to proceed more effectively in CD45RO+ cells. Binding of anti - CD45, anti - CD45RA and anti - CD45RO antibodies up - regulate binding through LFA - 1<sup>54, 65</sup> as does PKC activity stimulated by phorbol ester<sup>74</sup>. The increase in SF seen in these experiments after PdBu treatment or CD45 cross - linking occurred equally in both populations and was limited only by the onset of down - regulation of CD4 with PdBu<sup>60</sup>. The corollary of this is that inhibition of PKC reduces SF in both populations, although, as in the case of anti - CD18, CD45RO+ cells remain more efficient.

One reasonable explanation of these data is that in CD45RO+ cells increased LFA - 1 expression is accompanied by enhanced binding ability of this molecule, possibly because of the increased basal PKC activity in these cells<sup>61, 62</sup>. When PKC is stimulated by PdBu or CD45 cross - linking, SF is optimised in both populations. CD4 ligation by anti - CD4 or gp120 inhibits LFA - 1 mediated heterotypic binding<sup>75, 76</sup> particularly CD45RO+ cells<sup>56</sup>. But even when down - regulated after CD4 ligation, heterotypic binding is never less efficient in CD45RO+ cells. It is thus likely that enhanced heterotypic binding and SF ability mediated by LFA - 1 reflect part of an physiologically augmented adhesiveness of CD45RO+ cells.

Differences in LFA - 1 expression and affinity cannot be the whole explanation for the propensity of CD45RO+ cells to form syncytia, since these cells formed more syncytia than CD45RA+ cells in the presence of anti - CD18 and when PKC was inhibited. Binding through another, unidentified, set of adhesion molecules is up - regulated by signalling through TK<sup>55</sup> and appears to be regulated by CD45<sup>54, 55</sup>. When TK was inhibited by genistein there was a net reduction of SF with both populations performing equally poorly; CD45 cross - linking - known to activate TK<sup>63</sup>, - augmented SF equally well in both populations. This data suggest TK can regulate SF in these cells. CD45RO+ cells are not known to have higher basal levels of TK, but the data in the preceding chapter suggest CD45RO+ cells activate TK, particularly p56<sup>Lck</sup>, after gp120 binding and this may thus contribute to enhanced SF.

CD45 is known to increase heterotypic binding by PKC independent means through LFA - 1<sup>65</sup> and in monocytes transfected with p56<sup>Lck</sup> CD45 cross linking accelerates SF<sup>34</sup>. The data here suggest that p56<sup>Lck</sup> may activate adhesion molecules on CD45RO+ cells other than LFA - 1. The nature of the LFA - 1 independent, p56<sup>Lck</sup> regulated system awaits determination, although it is unlikely to be CD29, which although up - regulated on CD45RO+ cells, does not have its affinity regulated by CD45<sup>54</sup>. The HPBALL cells provide a suitable model to investigate whether these relevant molecules are integrins, selectins or members of the immunoglobulin super family.

The data from the HPBALL cells show that CD45 null cells form syncytia well and are more readily infected with HIV than their wild type counterparts. These data suggest that high molecular weight CD45 isoforms may exert negative effects on integrin and other molecule affinity, whilst CD45RO and the absence of CD45 confer neutral effects.

Although SF does not occur through the same mechanism as cell free virus fusion<sup>25</sup>, the majority of data suggest that integrins play an important role in both pathways<sup>41, 42</sup>. This assumes that virions can express integrin molecules, which may be plausible since they can passively take up MHC Class II molecules<sup>45</sup>.

SF not only contributes to viral spreading but also causes cytopathic effects *in vitro*, although failure to demonstrate syncytia *in vivo* more widely than in brain tissue<sup>77</sup> has cast doubt on this as a major pathogenic mechanism<sup>78</sup>. Whether the more rapid disease progression that occurs after the emergence of HIV strains *in vivo* which can induce syncytia *in vitro* is a direct consequence of increased viral load or cytopathic effect is unclear<sup>1, 3</sup>.

Although frequently used as an *in vitro* indicator of the efficacy of anti - HIV agents, SF has not always been clearly distinguished from viral replication, although these are distinct processes. SF can be completely inhibited in situations where viral replication continues. For example, PMA has been shown to decrease SF, in one system, whilst increasing viral replication, measured by p24 production<sup>59, 79</sup>. Similarly, it is clear from these data and those of others<sup>60</sup> that cell populations differ in their ability to support SF. This deserves consideration in *in vitro* assays of drugs.

HIV syncytium formation is not a process that is easily amenable to therapeutic intervention. Although best known for its effects on blocking the CD4/gp120 interactions, dextran sulphate also down - regulates LFA - 1 $\alpha$  and  $\beta$ , thus inhibiting HIV at multiple steps<sup>57</sup>. Dextran sulphate also inhibits the coagulation cascade but modifications have been made to the polysaccharide backbone which minimise this problem without affecting *in vitro* anti - HIV efficacy. It remains to be seen, from Phase I *in vivo* studies (J.Weber, personal communication), whether inhibition of adhesion molecule binding will cause toxicity.

On the other hand, immuno - restorative therapies, such as  $\gamma$ IFN, which is undergoing Phase III studies, also have the potential to up - regulate adhesion molecules such as ICAM<sup>46</sup> and may hence have unexpected detrimental effects.

Since during SF unintegrated viral DNA may spread from cell to cell, SF bypasses the need for reverse transcription and renders nucleoside drugs such as zidovudine ineffective<sup>60</sup>. Clinical evidence suggests that patients with syncytium inducing (SI) HIV strains may derive less benefit from zidovudine than those with non SI strains (unpublished data discussed in <sup>3</sup>).

CD45RO+ CD4+ T cells form syncytia perceptibly more readily than their CD45RA+ counterparts. This increased competence appears to be multifactorial; a consequence of increased integrin expression, integrin binding ability, binding through unidentified TK dependent molecules and possibly, due to its larger size, CD45RA

acting as an intercellular buffer. Whether the ability of CD45RO+ cells to fuse with cell free virus is augmented is explored in the next chapter.

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# Chapter 6. HIV Reverse Transcription And Integration In CD45RA+ And CD45RO+ CD4+ Lymphocytes.

Preferential infection of CD45RO+ CD4+ lymphocytes has been proposed as an explanation for the early loss of these cells in the natural history of HIV infection. Data show that HIV DNA is carried mostly in CD4+ cells expressing CD45RO<sup>1</sup> after *in vivo* and *in vitro* infection, but to date, no explanation of this preferential carriage has been offered. The experiments in this chapter aim to confirm this finding and establish an explanation.

## 6.1.1 HIV Reverse Transcription And Integration.

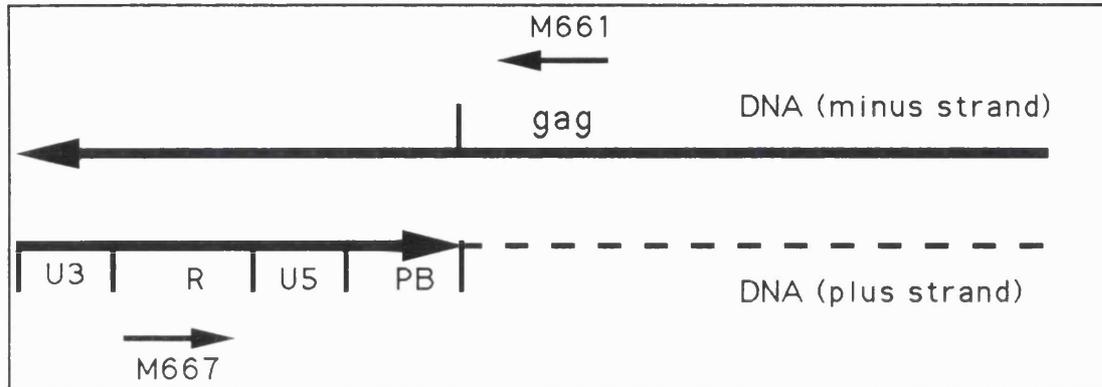
Following viral fusion and entry of RNA, HIV has minimal requirements to complete reverse transcription and integration of proviral DNA into the host genome. In the minimal model of erythrocytes transfected with CD4, reverse transcription (RT) of incoming viral RNA can be accomplished providing enough de - oxy - nucleotide - triphosphates (dNTPs) are available<sup>2</sup>. Hence, if CD4 is expressed and molecules involved in fusion and dNTPs are present, cells should be able to reverse transcribe viral RNA. So far, no inhibitory cellular factors have been identified.

A variant of this simple model is that RT may commence in a developing virion and that by the time a virus fuses with an uninfected cell it already contains a partially reverse transcribed genome<sup>3</sup>. This process can occur because reverse transcriptase packed in the developing virus is functionally mature and viral RNA is stabilised in the budding virion by zinc fingers in *gag*<sup>4</sup>. The extent of the intra - viral RT is also dependent on concentrations of dNTPs<sup>5</sup> and may also explain how zidovudine can give the impression of having partial post - transcriptional inhibitory effects on HIV<sup>6</sup> and conflicting data on the degree of RT in different cell lines.

Reverse transcriptase is a complex enzyme with at least 4 activities<sup>7</sup>; RNA dependent DNA polymerase, DNA dependent DNA polymerase, RNase, co - ordinated to lag 19 base pairs behind RNA dependent DNA polymerase degrading residual viral RNA, and a DNA polymerase<sup>8</sup> which mediates 2 strand transfers. The minus strand of DNA is primed by a tRNA primer binding to the 5' end of viral RNA and forms the upstream 5' repeat. This DNA is transferred to the 3' end, adopts a plus strand orientation and then acts as a template for a new minus DNA strand. The minus strand is completed (through genes for *env*, *pol* and finally *gag*) and the DNA template is transferred to its original position to allow completion of the plus DNA strand. This process ensures that the DNA genome is bound by two long terminal repeats (LTRs), required for transcriptional regulation (see next chapter). Primers used for PCR in this

chapter are anti - sense gag (661) and sense upstream repeat (667), which will hence only amplify when RT is well underway, after the second template switching event<sup>3, 9</sup>.

**Figure 6.1. Primers Detecting Late Reverse Transcripts.**



Following completion of RT, the proviral DNA exists in either circular or linear forms. Only the linear form is integrated into the host genome<sup>10</sup>, after a viral protein has cleaved the host DNA to prepare the receptor site and trimmed the ends of the proviral DNA<sup>11</sup>. This process is dependent on divalent cations but whether a cellular protein is also required remains controversial<sup>11, 12</sup>.

### 6.1.2. Cellular Activation And Reverse Transcription.

From the time of the initial discovery of HIV it was apparent that T cells require activation to establish active infection<sup>13</sup>. Early data<sup>14</sup> did not distinguish between stages up to and including integration from transcription, but it has subsequently become clear that both are upregulated by T cell activation. Transcriptional regulation of HIV is discussed in the next chapter.

Both integrated and unintegrated HIV DNA (high and low molecular weight forms) are found in T cells which have been activated prior to exposure to HIV<sup>15</sup>. When infection precedes activation it can be shown, using RT - PCR, that activation does not increase the amount of incoming viral RNA per cell<sup>9</sup>. When HIV DNA in the viral stock is eliminated using DNase, partially reverse transcribed RNA, although labile, can be rescued by mitogen stimulation several hours later, leading to successful RT<sup>16</sup>. Cells activated immediately after infection produce full length reverse transcripts, but when activation is delayed after infection, less viral DNA is produced, suggesting that in quiescent cells viral RNA is vulnerable to cellular RNAses<sup>9</sup>.

More detailed examination of events in quiescent cells, using conventional PCR with a series of primers which detect reverse transcripts at different stages of RT, showed that RT was occurring but was aborted early, around the first template switch<sup>16</sup>. Hence these partial reverse transcripts form a type of latent pool which is utilised on subsequent activation.

Abortive RT is probably not a consequence of a viral factor, since RT does not stop at discreet sites but is graded. A more likely alternative is that efficient RT requires the induction of a cellular co - factor (or removal of an inhibitor). Concentrations of dNTPs may be limited in resting cells and it may be that HIV reverse transcriptase, which has fastidious requirements for dNTP concentrations<sup>17</sup>, may exhaust dNTP stocks. The rate determining step in dNTP synthesis is the enzyme ribonucleoside reductase, which requires free oxygen radicals and is activated during T cell activation. Unpublished data show that reverse transcriptase is enhanced in resting T cells by the addition of dNTPs<sup>5</sup>.

The efficacy of RT can also be regulated by a viral factor, Vif. The presence of this factor broadens the spectrum of cells in which RT can proceed, although the mechanism for this activity remains unclear. Even though most wild type viruses express Vif, its activity is not potent enough to permit RT in quiescent cells<sup>18</sup>.

### 6.1.3. Integration Of HIV Provirus.

Integration of the HIV provirus into the host genome is required for optimal completion of the viral life cycle<sup>19</sup>. Hence integrase defective mutants, transfected into T cells do not produce infective progeny<sup>19, 20</sup>. The precise site of the block in unintegrated viruses is unclear, but probably lies at the level of transcription<sup>20,21</sup>, which does not precede effectively unless integration has taken place.

Stevenson found that HIV DNA only integrates once the host cell has been activated<sup>22</sup>. This was interpreted as suggesting both resting and activated cells can RT equally well but that there is a block to integration in resting cells<sup>22</sup>. An explanation for the difference between these data and those of Zack, is that Zack treated viral stock with DNase whilst Stevenson did not. Hence the low molecular weight HIV DNA detected by Stevenson may have come from incoming viruses. The possibility still remains that integration is more effective in stimulated cells.

In an extension of this work, it has been shown that the majority of infected T cells from individuals with AIDS contain high molecular weight HIV DNA, whilst the majority of infected cells from asymptotically infected individuals contain low molecular weight DNA. In both groups, high molecular weight DNA carriage is limited to cells expressing MHC Class II or CD25<sup>23</sup>.

Activation may result in more effective integration because of uncoiling of host DNA, the requirement for a putative cellular co - factor<sup>12, 22</sup> or more effective energy dependent transport of the unintegrated DNA to the nucleus<sup>23</sup>. This latter possibility is supported by the finding that *in vivo* activated T cells transport unintegrated DNA to the nucleus more efficiently than resting cells<sup>24</sup>.

The need for cell activation for RT and integration has led to the suggestion that cell cycling may also be important for these processes. Existing evidence argues against this; RT can take place in erythrocytes<sup>2</sup> and HIV infects non - cycling cells such as

macrophages<sup>25</sup> and cells arrested *in vitro*<sup>21, 26</sup>. Only one set of experiments have shown that cycling is required for integration and for cytopathic effect<sup>27</sup>.

Because the state of cellular activation affects processes involved in the late steps of the life cycle of HIV, the higher basal activation level of CD45RO+ cells<sup>28</sup> may explain the preferential *in vivo* infection that occurs in these cells<sup>1</sup>. Alternatively HIV may not preferentially infect all the CD45RO+ pool, but the subset of cells within it with increased expression of activation markers; expression of IL2R $\alpha$ <sup>29</sup> or MHC Class II<sup>23</sup> is associated with proviral carriage *in vivo*. It should be noted that the hypothesis that MHC Class II and IL2R $\alpha$  positive cells are the same has not been formally tested (see below).

To study the effects of activation *in vivo* and *in vitro*, CD4+ CD45RO+ cells were separated into Class II negative and positive prior to infection *in vitro*. Similarly, both CD45RA+ and RO+ expressing cells were infected with and without *in vitro* activation.

## 6.2. Results.

Fresh non - adherent PBMs were negatively selected using antibody cocktail and magnetic beads to enrich for CD4+ cells and then CD45RA+ and CD45RO+ populations. A proportion of cells from the latter population were depleted of MHC Class II positive cells using magnetic beads coated with anti - MHC Class II mAb (L243); these cells are referred to as CD45RO+ Class II-. The phenotypic analysis of typical separated populations, using FITC - mAb conjugates as indicated, are given in Table 6.1.

Table 6.1. Phenotypic Analysis Of CD45RA+, CD45RO+ Class II- and CD45RO Cells.

	CD45RA+	CD45RO+ Class II-	CD45RO+
CD4+ (%)	93.2	97.0	97.6
CD45RA+ (%)	91.2	8.7	9.4
CD45RO+ (%)	18.2	94.6	90.2
MHC Class II+ (%)	4.1	6.5	14.4
MHC Class II (median fluorescence)	1.11	1.49	1.76
IL2R $\alpha$ + (%)	6.2	17.2	15.8
IL2R $\alpha$ (median fluorescence)	1.67	2.64	2.27

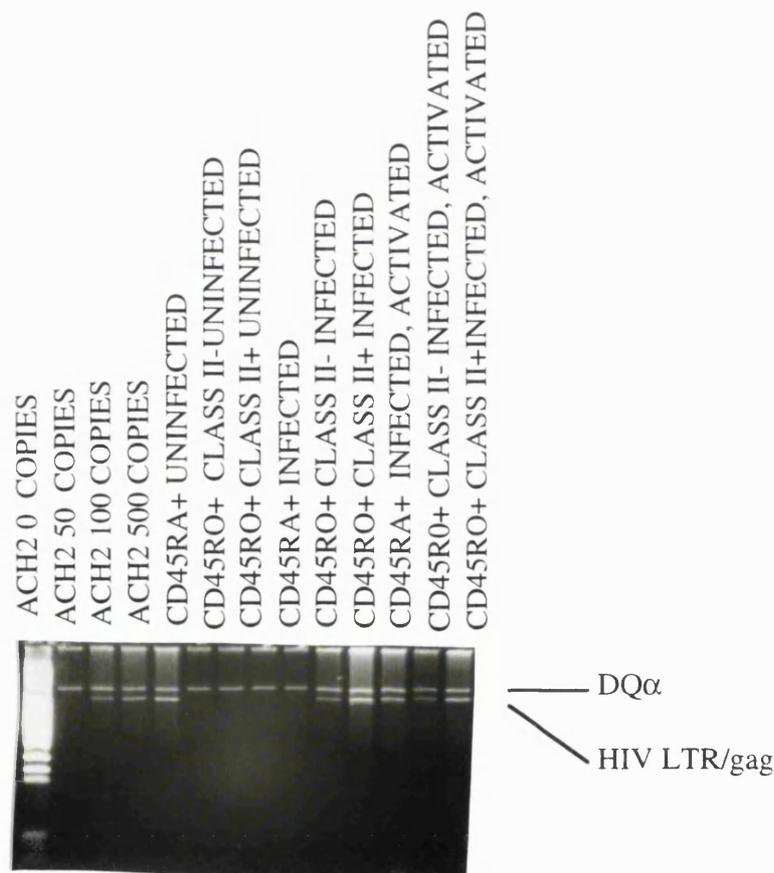
It can be seen in these data that CD45RA+ cells expressed less IL2R $\alpha$  and MHC Class II than CD45RO+ cells, as noted previously<sup>30</sup>. An unexpected but consistent

finding was that CD45RO+ Class II- cells expressed slightly more IL2R $\alpha$  than whole CD45RO+ cells.

Precautions were taken in these experiments to detect only newly reverse transcribed HIV DNA. Firstly, viral stock was treated with DNase to clear cellular HIV DNA associated with debris from cells that had undergone cytopathic effect<sup>9</sup>. Secondly, the primers used detect almost completely reverse transcribed DNA, which is carried at very low frequency in incoming HIV virions<sup>3</sup>.

In preliminary experiments (not shown) a 3 hour incubation with viral stock, washing of unbound virus, followed by a further incubation of 15 hours was found to give DNA signals in unseparated T cells. Although 40 cycles of PCR enabled 20 copies of HIV DNA to be consistently detected (not shown), 35 cycles were employed in these experiments and detected 50 copies of HIV DNA in ACH2 cells diluted in PBL.

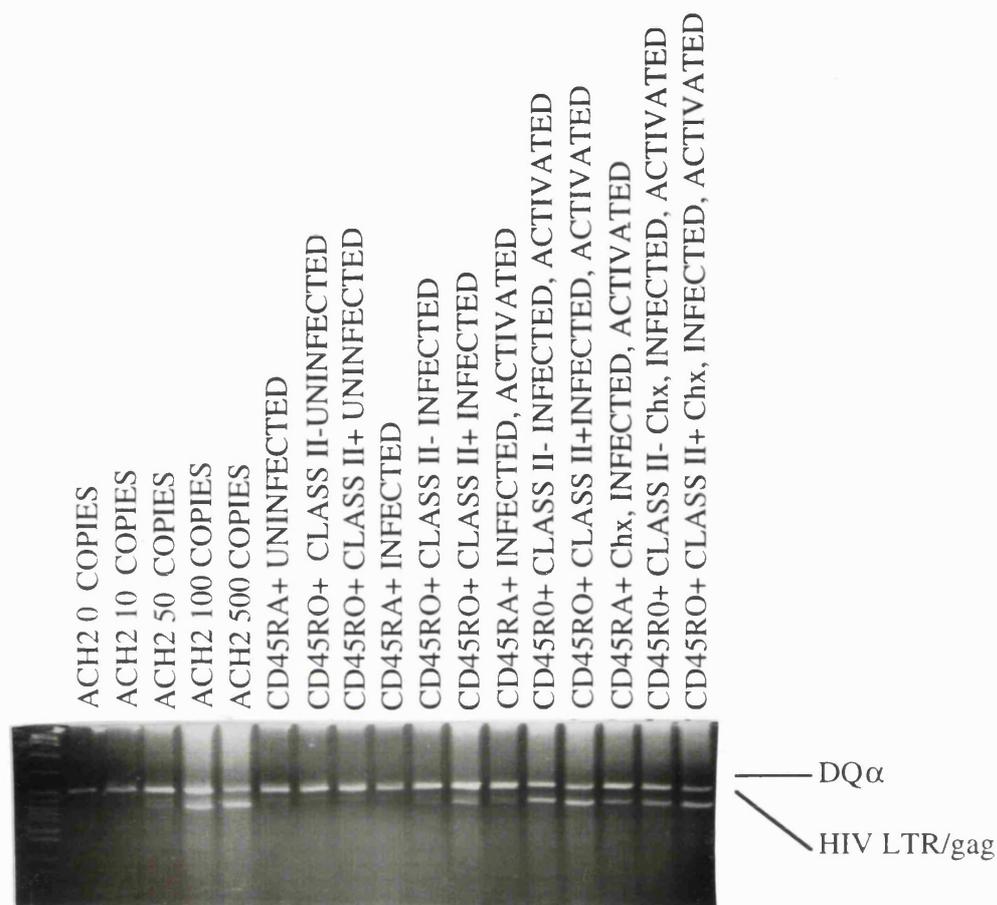
Figure 6.2. HIV DNA In CD45RA+ And CD45RO+ T Cells After *In Vitro* Infection.



In the experiment shown in Figure 6.2 an MOI of 0.125 was used. All lanes contain bands corresponding to DQ $\alpha$ , suggesting that DNA extraction was satisfactory and that no preparations contained PCR inhibitors. Fifty copies of HIV are detected in ACH2 cells. Uninfected separated PBL cells contain no HIV DNA.

Infection followed by 15 hours incubation at rest resulted in infection of CD45RO+ Class II- and CD45RO+ Class II+ cells only, i.e. at least 50 HIV copies per  $2.5 \times 10^5$  cells. CD45RA+ cells did not yield a detectable band and so contained less than 50 copies of HIV. When cells were infected, washed and then incubated in the presence of PHA  $5\mu\text{g/ml}$  and PMA  $5\text{ng/ml}$ , all populations contained detectable copies of HIV DNA.

Figure 6.3. HIV DNA In CD45RA+ And CD45RO+ T Cells After *In Vitro* Infection.



The experiment shown in figure 6.3 is typical of those carried out at a lower MOI, in this case 0.0625. PCR barely detects 50 copies of HIV DNA in ACH2 cells. There is no detectable HIV DNA in uninfected separated PBL cell. Following infection without stimulation a barely visible HIV DNA band is present in CD45RO+ ClassII- and a clear band present in CD45RO+ ClassII+ cells. When infection was followed by activation, the band in CD45RO+ ClassII- cells becomes more distinct whilst a dim band in the CD45RA+ cells appears.

To assess whether increased RT after *in vitro* activation involves *de novo* protein synthesis, some populations of cells were incubated with cycloheximide (Chx) 10mM for 1 hour, between infection and activation (viability remained greater than 95%, trypan blue). Inhibition of protein synthesis with Chx did not inhibit the activation associated increase in HIV DNA in CD45RO+ Class II- and CD45RA+ cells.

## 6.3. Discussion.

Two practical points ensured the validity of these data. In these experiments, as in those of Zack<sup>9</sup>, viral stock was treated with DNase and primers were used that detected almost completely reverse transcribed DNA. Hence contaminating incoming virion DNA does not confuse the results; viral DNA detected is very likely to be newly reverse transcribed. This is inferred by the absence of HIV DNA in resting CD45RA+ cells, which can bind gp120 (chapter 3). Secondly, the PCR reaction conditions and the amplificate detection system were set so as not to be too sensitive. Although systems exist which claim to be able to routinely detect single copies of HIV DNA in biological material<sup>31, 32</sup>, these may have been too sensitive for this experimental system when the CD45 expressing T cell populations could have been contaminated with their reciprocal CD45 population (see Table 6.1).

Depleting CD45RO+ cells using magnetic beads indirectly coated with anti - MHC Class II reduced Class II expression. IL2R $\alpha$  was measured on these populations, originally as a collateral test of removal of *in vivo* activated cells. Unexpectedly, CD45RO+ Class II- cells expressed rather more IL2R $\alpha$  than undepleted CD45RO+ cells. Both MHC Class II and IL2R $\alpha$  are upregulated after activation of CD45RO+ cells<sup>30</sup>; these data infer that both “activation markers” may not be upregulated synchronously on the same cells, although this was not tested formally.

These data show that HIV infection *in vivo*, without activation occurs preferentially in CD45RO+ cells. Within CD45RO+ cells, when MOI and PCR efficacy are limiting, infection preferentially occurs in the Class II+ subset of CD45RO+ cells. Hence these data confirm and extend those of Schnittman<sup>1</sup> and Stevenson<sup>23</sup>, that HIV preferentially infects CD45RO and MHC Class II+ cells, respectively. These authors have shown that the same selectivity occurs *in vivo* and *in vitro*.

Anti - MHC Class II antibodies can inhibit syncytia formation<sup>33</sup>, thus the CD45RO+ Class II- cells could contain less DNA because viral fusion was inhibited by residual antibody used in the preparation of these cells. This is an unlikely explanation since subsequent activation could rescue these cells.

These data show that the block to the completion to the HIV life cycle in Class II- and CD45RA+ cells can be overcome *in vitro* with activation after infection. These experiments do not discern differences in integration in the populations studied, since whole cellular DNA was used in the PCR reaction. In this system, protein synthesis inhibition did not inhibit the increase in viral DNA after activation.

One demonstrated reason for impaired RT in resting T cells is diminished reserves of dNTPs<sup>17</sup>. These are synthesised by an inducible enzyme, nucleotide reductase, which is inhibited by hydroxyurea<sup>5</sup>. Hydroxyurea is too toxic for routine treatment of HIV infection, but may provide a model for new therapies<sup>5</sup>. Importantly, nucleoside analogues such as zidovudine ddI and ddC do not require ribonucleoside reductase to inhibit reverse transcriptase<sup>34</sup> and so may inhibit RT in resting cells with

limited dNTP pools. Whatever processes enhance RT after cell activation, it is clear that CD45RA+ cells can be infected with HIV in appropriate conditions. Since exposure to virus preceded washing and activation, it is clear that binding, fusion and RNA entry can take place in these cells before activation. However, there appears to be diminished quantity of DNA in these cells, even after activation, suggesting one of the early processes is impaired. The data in the previous chapter suggest this may be due to defective virus fusion in the CD45RA+ population.

Compounds have also been identified with activity against HIV integrase<sup>35</sup>. An alternative therapeutic strategy may be to clear activated cells, which may contain provirus. This has been done successfully *in vitro* with ricin linked anti CD25 antibody<sup>29</sup>, although would have an immunosuppressive effect of its own if used *in vivo*.

Un - reverse transcribed HIV RNA and un - integrated DNA are relatively short lived latent forms of HIV, functionally similar to integrated DNA<sup>36</sup> which also requires T cell activation for the viral life cycle to be completed. These forms of latency only operate in quiescent cells in which the mechanisms for RT and integration are not optimal. It would appear that CD45RA+ cells and CD45RO+ not expressing Class II are not activated sufficiently for RT to be fully functional; enough activation to upregulate expression of Class II or CD25 appears to be required for RT to be completed. Expression of CD25 implies commitment to activation<sup>37</sup> and, in CD45RA cells, switching to CD45RO<sup>30</sup>. Hence, CD45RA cells infected with HIV but maintaining latency with incomplete RT or integration can be rescued by activation, but in doing so are likely to become CD45RO+.

This could resolve the finding that HIV can infect CD45RA+ cells *in vitro*, but these are rarely found to carry HIV DNA<sup>1</sup> or produce p24 (after activation)<sup>38</sup> in *in vivo* HIV infection. This argues against frequent reversion of CD45RO+ cells to CD45RA+, since in this model HIV DNA is a marker of transient CD45RO expression.

Infected leukocytes from brain tissue of AIDS dementia patients occasionally contain multiple unintegrated HIV DNA and show cytopathic change<sup>39</sup>, the result of superinfection<sup>40</sup>. One model for the pathogenesis of AIDS is that unintegrated provirus is cytopathic for T cells<sup>41,42</sup> as occurs with more cytopathic retroviruses. This is inconsistent with the finding that, *in vitro*, T cell lines can contain large numbers of unintegrated provirions without cytopathic effect<sup>40,43</sup> and that *in vivo* infected cells contain only one HIV DNA copy per cell<sup>44,45</sup>, presumably due to effective superinfection control (HIV mediated downregulation of CD4)<sup>33</sup>.

In summary, unstimulated CD4+ cells show a continuum of basal activation, which is reflected in their ability to reverse transcribe HIV. CD45RO+ Class II+ cells perform RT well, perhaps because of their higher levels of dNTPs. CD45RA+ cells show diminished HIV DNA, even after post - infection activation, perhaps reflecting impaired fusion.

The major stable form of HIV latency occurs in recently activated CD45RO+ Class II+ cells. How this may be broken is discussed in the next chapter.

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# Chapter 7. HIV Latency And TNF Mediated Signals In CD45RA+ And CD45RO+ CD4+ T Lymphocytes.

## 7.1.1 The Nature Of Viral And Clinical Latency.

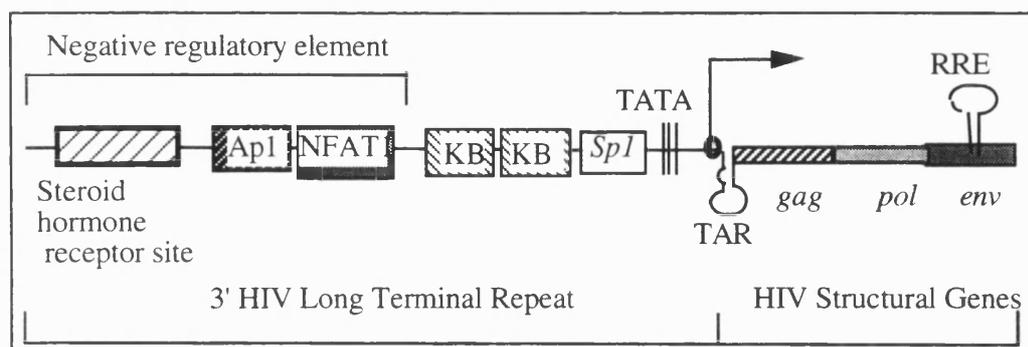
Clinical latency is the state of asymptomatic carriage of HIV and represents the balance of diminished viral replication and adequate host immune response<sup>1</sup>. During this stage, however, both viral and immunological markers can suggest clinically silent but progressive viral replication, particularly in lymphoid tissue<sup>2</sup>, along with impairment of the immune response. Following seroconversion illness, when viral replication is abundant<sup>3</sup>, there is a quiescent phase representing the onset of immunity which inhibits HIV transcription rather than clears infected cells<sup>4</sup>. In this phase of viral latency, antigenic stimulation, as a result of intercurrent infection<sup>5</sup> or vaccination<sup>6</sup>, transiently up - regulates HIV replication. If HIV proteins act as super - antigens<sup>7, 8</sup> or triggers for auto - immunity<sup>9-14</sup> these may contribute to generalised T cell activation and enhanced HIV replication. Subsequently, a progressive increase in HIV transcription, detected as mature RNA<sup>15</sup> or viral antigen<sup>16</sup> occurs. This increase in replication may represent, in part, evolution of escape mutants<sup>17, 18</sup> and an unexplained decline in the CTL response<sup>19, 20</sup>.

During viral latency, reverse transcribed HIV is integrated into the cellular genome, but there is no transcription. At a cellular level, HIV may be maintained in an untranscribed state by DNA methylation<sup>21</sup>, through failure of integration<sup>22</sup>, or by abnormal RNA splicing<sup>23</sup>. These situations appear to be unusual, however, and in most cases the replication of HIV is regulated tightly by the interaction of cellular transcription factors with the long terminal repeat (LTR).

## 7.1.2. The Regulation Of HIV Transcription.

The essential elements regulating HIV transcription are the LTRs which bind cellular factors, the TAR region binding Tat and cellular factors and the Rev response element (RRE), contained within the *env* gene, responsible for switching transcription from regulatory to structural genes. These are shown in figure 7.1. HIV contains enhancing sites within the genome; a  $\kappa$ B site has been identified in the *tat* and *rev* genes, although it is not clear how functional it is<sup>24</sup>.

Figure 7.1. The HIV Long Terminal Repeat.



### 7.1.3. The HIV LTR And Cellular Transcription Factors.

The first cellular protein found to bind the HIV - LTR was Sp1, which mediates basal transcription<sup>25-28</sup> and facilitates upregulation by NFκB<sup>29</sup>, HIV Tat binding<sup>30, 31</sup> and trans - acting proteins of other viruses<sup>32</sup>.

Cellular proteins constitutively bind to the TATA site<sup>25</sup>, deletion of which results in reduced basal transcription<sup>27, 33</sup>. Whether activation through the TATA site takes place depends on the cellular proteins available. Hence binding of TFIID to TATA permits transcription, whilst induction of the factor UBPI inhibits it<sup>34</sup>. Proteins from other viruses also induce HIV through the TATA box<sup>32, 35</sup>.

The HIV - LTR contains tandem κB motifs which are bound by T cell proteins on activation by PHA<sup>36</sup>, phorbol ester, anti - CD3, anti - CD28<sup>37,38</sup>, antigen<sup>39</sup>, anti - CD2<sup>40</sup>, TNF<sup>41-43</sup>, IL1<sup>43</sup>, ultra violet light<sup>44</sup>, heat shock<sup>45</sup>, HIV Tat<sup>30</sup>, HTLV1 Tax<sup>46,47</sup> and Herpes virus regulatory proteins<sup>48,49</sup>. CD45 has inhibitory effects on NFκB induction and HIV transcription - mutant T cells not expressing CD45 have constitutive NFκB activity<sup>50</sup>. The majority of the data below come from T cell lines where NFκB induction mirrors HIV - LTR reporter gene activation and HIV transcription; in T cell clones this is not always the case<sup>39</sup> and there are no data on NFκB induction and HIV transactivation in fresh T cells.

Stimuli induce NFκB using different second messengers, so the effects of TNF, for example, may be additive to those of phorbol esters<sup>43,51</sup>. PKC activation is a pre requisite for both pathways<sup>52</sup> although calcium influx is not required<sup>53</sup>; hence signals that activate HIV through NFκB are not necessarily mitogenic<sup>53</sup>. Phorbol ester and TNF additionally induce NFκB via ROIs<sup>54</sup> and hydrogen peroxide can induce HIV transcription *in vitro*<sup>55</sup>. IL1 uses PKA as a second messenger in the induction of NFκB<sup>56-58</sup>.

These second messengers induce the Rel family members HIVEN86<sup>59</sup> and NFκB p50<sup>60</sup>, which bind the κB sites in the HIV - LTR, resulting in transcriptional upregulation<sup>25</sup>. Deletion of the κB motifs results in impaired response to stimulation but does not abolish transcription totally, unless the Sp1 site is also abolished<sup>29</sup>.

NFκB and HIV transcription are induced in immature monocytes by phorbol ester, TNF<sup>61</sup>, CD14 ligation<sup>62</sup> by LPS<sup>63</sup> and forced maturation by adhesion<sup>61</sup> or retinoic acid<sup>64</sup>; NFκB activity is constitutive in mature macrophages.

The κB sites are highly conserved across HIV strains<sup>65</sup> and spontaneous mutations in the HIV κB sites modify the replication rates, pathogenicity and tendency to develop latency<sup>66-68</sup>. The enhancer of HIV - 1 is more active than that of HIV - 2, possibly because HIV - 2 has only one κB site while HIV - 1 has two<sup>69</sup>. This may explain the reduced pathogenicity of HIV - 2 *in vivo*<sup>70</sup>.

HIV induces positive feedback of its own transcription by inducing NFκB<sup>71</sup>, by induction of p105 transcription<sup>72</sup>, HIV protease mediated lysis of the p105 precursor to the active p50 peptide<sup>73</sup> and possibly by inducing maturational changes whereby transcription factors are expressed constitutively<sup>74</sup> - at least in monocytes. Balancing the effects of HIV on NFκB is the viral regulatory peptide nef, which inhibits NFκB induction<sup>75</sup>, possibly by uncoupling NFκB from the T cell receptor mechanism<sup>75</sup>.

HIV κB sites may not be important in transcriptional regulation in all systems. Recombinant HIV and SIV strains with no κB sites were able to replicate in human<sup>29</sup> and rhesus T cells<sup>76</sup>, respectively. In the latter experiment, SIV replication in rhesus macrophages was impaired<sup>76</sup>, suggesting that the κB sites may be involved in tropism<sup>65</sup>. In transgenic mice, where envelope determined tropism is no longer relevant, HIV - LTR expression is highest in dendritic cells in skin<sup>77</sup> and primary lymphoid tissues<sup>78, 79</sup>. These data suggest that transcription through the HIV - LTR is different in some tissues, although generally eclipsed by envelope tropism, and may also explain the absence of NFκB mediated transcription in some cells.

Like the IL2 LTR, HIV - LTR contains a transcriptionally active NFAT site upstream of the κB motifs<sup>80</sup>. Binding to the NFAT motif is an absolute requirement for IL2 transcription, whilst in HIV, NFAT only enhances NFκB mediated induction<sup>37, 81</sup>, making the HIV - LTR functionally similar to IL2Rα<sup>37, 82</sup>. Consequently HIV transcription is resistant to cyclosporin A<sup>83</sup>.

Upstream of NFAT is an AP - 1 site<sup>84</sup>, although it is not clear whether this is transcriptionally active. The AP - 1 and NFAT sites lie within a negative regulatory element (NRE), deletion of which enhances transcription<sup>25, 85</sup>, although this has not been universally observed<sup>33</sup>. This is structurally similar to an NRE in the IL2Rα LTR<sup>86, 87</sup>; both NREs bind a constitutively expressed protein<sup>88, 89</sup> which mediates inhibition. Within the NRE is a site homologous to the steroid hormone receptor binding sites<sup>90</sup> which binds *in vitro* to this family of proteins<sup>84</sup>. Furthermore, T cells contain a novel 100kD protein which binds to this site<sup>91</sup>, the significance of which is unclear since 1, 25 - di - hydro - cholecalciferol up - regulates HIV transcription in acutely infected cells<sup>92</sup> whilst dexamethasone inhibits phorbol ester mediated transcriptional upregulation<sup>93</sup>. This site is not intact in all strains, which may explain inconsistencies in detecting NREs and in HIV strains<sup>84</sup>. Finally, the negative regulatory factor of HIV, nef, may bind the NRE region<sup>94</sup>(see below).

### 7.1.4 TAR, Tat And Viral Regulatory Factors.

The HIV trans - activating protein, Tat, regulates the basal processivity of transcription<sup>36</sup> and co - ordinates up - regulation through the  $\kappa$ B site<sup>95</sup>. Deletion of the *Tat* gene or its binding site, TAR, results in diminished transcription<sup>29</sup>. TAR forms a stem/loop structure; Tat binds a side bulge in the stem and a cellular protein<sup>27</sup>, UBP1, binds the loop<sup>96</sup> and adjacent sites in the TATA box<sup>33</sup>. Tat is one of the first HIV proteins to be transcribed<sup>15</sup> and Tat and UBP1 bind onto TAR RNA, requiring a degree of transcription before this mechanism operates<sup>30</sup>. Without the TAR/Tat/UBP1 complex, transcription is abortive and produces short, random length transcripts. Spontaneous changes in Tat may account for some of the inter - strain variations in virulence in HIV (reviewed in<sup>1</sup>). TAR is also implicated in the transactivation mediated by viral products from HSV<sup>35, 97</sup>, HHV6<sup>49, 98, 99</sup> and EBV (see below).

The *Tat* gene has diverse other effects<sup>100</sup>, for example it has positive post transcriptional effects on HIV proteins<sup>101</sup>, up - regulates TNF secretion<sup>102, 103</sup>, acts as a growth factor for Kaposi's sarcoma<sup>104, 313</sup>, down - regulates CD4<sup>105</sup> and possibly inhibits T cell proliferation<sup>106, 107</sup>, although it does not affect T cell tyrosine phosphorylation<sup>108</sup>. The potential role of Tat as a growth factor for Kaposi's sarcoma is particularly interesting; Tat uses integrins as a receptor on endothelial cells and, using fibroblast growth factor as a co - factor, stimulates endothelial cell proliferation<sup>104, 313</sup>.

HIV negative factor, Nef, was so named because it was shown to inhibit HIV - LTR reporter gene transcription *in vitro*<sup>94</sup>, although these effects proved impossible to repeat. Nef has been shown to bind the HIV NRE in some studies<sup>94, 109</sup>, although this finding has not been universal<sup>110</sup>.

Nef has negative effects on other genes, inhibiting IL2 and, possibly, NF $\kappa$ B induction<sup>75</sup>. *In vitro* Nef down - regulates CD4 expression<sup>111, 112</sup> and depletes CD4+ thymocytes in transgenic mice<sup>112</sup>. The finding that Nef requires myristoylation to function<sup>109</sup> supports a role at the cell surface and explains the variable effects of Nef isolates, since the myristoylation site is frequently non - functional<sup>111</sup>. One interpretation of these data is that Nef impairs a step in signal transduction<sup>110</sup>. In this model Nef has no direct effects on HIV transcription but inhibits the immune response to HIV *in vivo*. Infection of monkeys with Nef deleted SIV results in diminished viral replication<sup>113</sup>. If these findings are reproducible in humans, Nef - deleted HIV strains may have potential as live vaccines.

To complete its life cycle, HIV sequentially produces 3 species of RNA; 1) multiply spliced 2kB RNA for the regulatory genes products Tat, Rev and Nef, 2) singly spliced 4.5kB RNA for env, Vif, Vpu and Vpr, 3) unspliced, for gag, pol and genomic RNA. Viruses defective in the Rev gene produce predominantly 2kB species and are non - viable<sup>23</sup>. Rev switches splicing to the higher molecular weight message<sup>114</sup>, acting as a negative regulator of its own synthesis and promoting maturation. Asymptomatic HIV infected patients produce mainly 2 and 4.5kB HIV RNA, not producing unspliced

RNA until the onset of symptomatic disease<sup>15</sup>, suggesting that in this case viral latency mirrors clinical latency.

### 7.1.5. Other Viruses And HIV Transcription.

Individuals infected with HIV are frequently hosts to other organisms transmitted by the same route of infection; for example, Cytomegalovirus (CMV) and Hepatitis B infection are consequences of “unsafe sex” and intravenous drug use, along with HIV. Intercurrent infections have the potential to activate HIV in a number of ways.

Intercurrent infection increases HIV transcription in CD4+ T cells responding to TcR stimulation<sup>39</sup> or to polyclonal activation by cytokines<sup>115</sup>. In monocytes, LPS released from Gram negative organisms<sup>63</sup>, double stranded viral RNA<sup>116</sup> and intracellular Mycobacteria<sup>117</sup> all induce HIV transcription. Viruses capable of infecting the same target cells as HIV may be capable of upregulating HIV transcription as follows.

Epidemiologically, co - infection with HTLVI and HIV confers a worse prognosis than HIV alone<sup>70, 118-120</sup> and co - infection of T cells with HTLVI increases HIV replication<sup>121</sup>. HTLVI encodes a protein, Tax, which promotes binding to the  $\kappa$ B motif in the IL2<sup>122, 123</sup>, IL2R $\alpha$ <sup>86, 87, 89</sup> and HIV<sup>47, 85</sup> LTRs, possibly explaining these effects. The up - regulation of growth factor/receptor genes is involved in the oncogenic potential of HTLVI<sup>124</sup> and tumours in *Tax* transgenic mice have remitted after treatment with anti - sense NF $\kappa$ B p50 and p55 nucleotides<sup>125</sup>. The mode of action of HTLV1 Tax on NF $\kappa$ B is unclear, although Tax promotes HTLV1 transcription through a different pathway, involving *cfos*<sup>46</sup>.

CMV frequently co - infects with HIV<sup>126</sup> resulting in a measurably worse prognosis than HIV alone<sup>127-130</sup>. CMV induces macrophage cytokine secretion, with subsequent effects on the HIV - LTR<sup>131</sup> and also transactivates the HIV - LTR directly<sup>132</sup>. This involves co - operation of cellular<sup>133</sup> and CMV proteins acting independently of the  $\kappa$ B sites<sup>32,97,134</sup> and is specific for HIV<sup>135</sup>.

Although not been linked to worse prognosis in HIV infection<sup>126</sup>, herpes simplex transactivates the HIV - LTR<sup>97</sup> using a similar mechanism to CMV<sup>35</sup> and by inducing NF $\kappa$ B binding<sup>48</sup>. Epstein Barr virus infection makes B cells permissive to HIV infection and transactivates the HIV - LTR *in vitro*<sup>136</sup>, operating both diffusely across the LTR<sup>134</sup> and by inducing NF $\kappa$ B<sup>137</sup>. Human herpes virus 6 transactivates the HIV - LTR and induces NF $\kappa$ B binding<sup>49, 98, 99</sup>, but does not affect prognosis<sup>138</sup>. Adenovirus<sup>35</sup> and Hepatitis B virus<sup>139</sup> both transactivate HIV, but have no clinical “co - factor” effects<sup>140</sup>.

### 7.1.6. Cytokine Abnormalities In HIV Infection And Their Effects On HIV Transcription.

Studies on cytokine secretion in HIV infection show conflicting data depending on the type of clinical samples and techniques used. Macrophages acutely<sup>141, 142</sup> or

chronically<sup>143</sup> infected with HIV *in vitro* have been shown to secrete increased amounts of IL1, although when meticulous attention has been paid to excluding LPS, production of IL1 appears to be normal<sup>144-146</sup>. IL1 is also secreted when monocytes are exposed to T cell derived<sup>147</sup> but not recombinant gp120 (rgp120)<sup>144,145</sup>. On the other hand, monocytes exposed to heat<sup>148</sup> or UV<sup>149</sup> treated HIV have shown decreased IL1 secretion.

HIV infection does not increase mononuclear cell IL6 or GMCSF secretion<sup>141, 144,146</sup> although gp120 exposure may do so<sup>147</sup>.

Macrophages infected with HIV *in vitro* also show increased TNF secretion<sup>141, 142</sup>, but again, not when conditions with minimal LPS are employed<sup>144-146,150,151</sup>. Exposure of macrophages to heat treated HIV<sup>148</sup> or T cell gp120<sup>147</sup> have been shown to increase TNF secretion. In other experiments rgp120 had no such effects<sup>144, 145</sup>. *In vitro* infection of macrophages<sup>141, 145, 152</sup> and T cells<sup>153</sup> decreases  $\gamma$ IFN secretion.

Although infection of T cells does not appear to result in increased TNF secretion<sup>151</sup>, exposure to rgp120 may do so<sup>154</sup>. B cells have also been found to secrete TNF when exposed to gp120<sup>155</sup>. Studies on TNF in blood or mononuclear cells infected *in vivo* have been equally confusing and are tabulated in Table 7.2.

Although it has been proposed that HIV infection up - regulates TNF transcription by inducing NF $\kappa$ B (see section 7.1.3. above) or through Tat<sup>102</sup>, these conflicting data suggest this is not a dramatic effect. TNF or IL1 secretion following infection or exposure to inert HIV peptides<sup>144, 145</sup> may occur because of the inadvertent presence of LPS or cellular debris. Differences in the maturity of the monocytes<sup>144, 145</sup>, the presence of soluble TNF receptors<sup>181, 182</sup> or an IL1 antagonist<sup>183</sup> when bio - assays have been conducted may explain some of the variations in these data.

These *in vivo* data suggest TNF and IL1 are raised during early HIV infection, when intercurrent opportunist infections (OIs) cannot be the cause. Subsequently OIs may trigger TNF and IL1 secretion, confounding the picture in late infection; when patients with overt OIs are excluded, late infection is not associated with increased TNF or IL1 secretion. Since HIV replication, at least in blood, is reduced in early HIV infection<sup>184</sup> it cannot necessarily be inferred that HIV induces cytokine secretion directly.

An equally plausible explanation is that clones of T cells responding to HIV contribute to the increase in TNF, so long as the immune response persists. CD8+ T cells responding to HIV produce soluble factors with both lytic and non - lytic anti - HIV activity<sup>185,186</sup> in Class I restricted and unrestricted manners<sup>186</sup>. The factors responsible for the lytic effects appear to be TNF and  $\gamma$ IFN<sup>187</sup> and although the non - lytic factor remains unidentified<sup>188,189</sup>,  $\gamma$ IFN remains a candidate<sup>159</sup> (TNF has only been shown to inhibit HIV replication non - lytically in non - lymphoid cells<sup>190</sup>). CD4+ T cells can respond to HIV peptides with an increase in membrane bound TNF, although this does not inhibit HIV<sup>191</sup>. Soluble TNF and  $\gamma$ IFN induce apoptosis in HIV infected cells<sup>169, 192, 193</sup>, which express increased TNFR<sup>181</sup>.

Table 7.2. Cytokine Abnormalities In HIV Infection.

	Clinical Situation	Assay	Reference
αIFN	Raised in symptomatic HIV infection.	Serum bioassay, RIA.	156, 157
	Raised in early HIV infection.	Message.	158
γIFN	Decreased at all stages of <i>in vivo</i> infection.	T cell supernatant and message.	153, 159-161
IL1	Raised in early asymptomatic HIV infection.	Serum ELISA.	16, 162
	Raised in early asymptomatic HIV infection.	Mφ supernatant.	103, 163
IL4	Increased secretion.	T cell clones, fresh PBL.	164, 165
	Decreased at all stages of <i>in vivo</i> infection.	Supernatant and message.	159, 160
IL6	Increased at all stages of HIV infection.	T cell clones.	103, 166
TNF	Raised in late HIV infection.	Message.	158
	Not raised at any stage.	Serum ELISA.	167
	Raised throughout HIV infection (OIs included).	Serum ELISA.	157
	Raised at all stages, especially symptomatic HIV infection; (OIs included).	Plasma ELISA.	168, 169
	Raised early, but not in symptomatic HIV infection (OIs excluded).	Serum ELISA.	162, 170
	Raised at all stages of HIV infection, especially during OI.	Alveolar Mφ, PBM.	171-175
	T cell surface TNF increased, especially in AIDS.	Flow cytometry.	176
	Increased secretion by alveolar and blood derived macrophages exposed to <i>P. Carinii</i> .	Serum ELISA.	177, 178
	Not elevated in paediatric AIDS, except in lymphocytic interstitial pneumonitis.	Serum ELISA.	16
	Raised particularly in context of TB with HIV.	Serum ELISA.	179
AZT had no impact on raised levels in symptomatic patients; OIs not excluded.	Serum ELISA.	180	

OI: opportunist infection. ELISA: enzyme linked immuno - sorbent assay.

Levels of circulating TNFR are increased progressively in HIV infection<sup>167, 182</sup>, possibly as a consequence of HIV infection of CD4+ T cells<sup>181</sup> or of non-specific lymphocyte activation, since acid labile IFN also induces TNFR<sup>194</sup>.

Reduced levels of glutathione and increases in glutamine in blood and tissue fluid are a feature of HIV<sup>195, 196</sup> and SIV<sup>197</sup> infection. Although there are no progressive changes in glutamate and glutathione during HIV infection, levels do relate to circulating CD4 counts<sup>198</sup>. These metabolic changes are thought to be due, at least in part, to raised TNF secretion<sup>199, 200</sup> and can potentiate immunosuppression directly<sup>201, 202</sup> as well as enhancing *in vitro* HIV replication in T cells and macrophages<sup>203, 204</sup>. Consequently, anti-oxidants have received considerable attention as potential HIV therapies<sup>199, 200</sup> (see below).

Increased TNF secretion has other potent effects and has been implicated in the neuronal damage of HIV encephalopathy when secreted by infected microglial cells<sup>142</sup>, B cell hyperplasia and polyclonal gammopathy<sup>176</sup>, as a growth factor effects to Kaposi's sarcoma<sup>104</sup>, myelosuppression<sup>205, 206, 314</sup> and in contributing to weight loss<sup>207</sup>.

### 7.1.8. Effects Of Cytokines On HIV Transcription.

Supernatant from macrophages exposed to LPS, HIV or other viruses stimulates HIV replication in T cell lines, an effect abrogated by anti-TNF<sup>131, 208</sup>. rTNF increases production of HIV proteins and cytopathic effects in chronically infected T cell lines<sup>51</sup> and increases SF in freshly infected PBL T cells<sup>209</sup>. TNF up-regulates HIV transcription<sup>42</sup> by increasing NFκB binding<sup>41, 43, 86, 89</sup>. The effects of TNF may be augmented by HIV mediated upregulation of TNFR expression<sup>182</sup>. Exogenous TNF initiates a prolonged phase of increased TNF secretion, which may then be self-maintaining HIV replication<sup>210</sup>. Hence, TNF has dichotomous effects on HIV infected T cells; on one hand it can upregulate HIV transcription, but on the other it causes lysis of infected cells. Which of these events is favoured *in vivo* remains unclear. Interestingly, anti-Fas induces apoptosis of HIV infected T cells, but unlike TNF, it has no effects on HIV transcription<sup>211, 212</sup>.

In monocytes HIV replication is increased by soluble<sup>213</sup> and membrane bound TNF<sup>214</sup>. TNF also increases HIV replication in glial cells<sup>215</sup>.

γIFN has complex effects on HIV infected cells. It does not induce HIV-LTR reporter genes<sup>43</sup> but does somehow increase HIV replication in macrophages<sup>216</sup>. Since immune serum is required for this positive effect, it may be mediated in part by increasing FcR expression<sup>217</sup>. On the other hand, γIFN decreases HIV replication in T cells and may induce apoptosis<sup>192</sup>.

IL6 induces HIV by transcriptional and post-transcriptional mechanisms<sup>213</sup> and, although *in vitro* data suggest IL6 is not induced by HIV infection, levels appear to be raised *in vivo*. Granulocyte-macrophage-colony-stimulating-factor (GM-CSF) does not stimulate NFκB reporter gene activity<sup>43</sup> but does induce HIV replication in macrophages<sup>218</sup> and monocytes<sup>213</sup>. This is mediated by inducing monocyte

maturation<sup>23</sup>, by upregulating CD14<sup>62</sup> and by altering the splicing pattern of HIV RNA<sup>63</sup>. rGMCSF, but not granulocyte - colony - stimulating - factor (rGCSF), increases p24 production from *in vivo* infected CD34+ bone marrow cells<sup>219</sup> and although rGMCSF can relieve the neutropenia associated with cytotoxic drugs in HIV infection<sup>220</sup>, it can increase blood p24 levels<sup>219</sup>. rGCSF has not been noted to have these *in vivo* effects.

### 7.1.8 Feedback Loops In HIV Transcription Regulation.

The majority of data presented in the preceding sections are derived from T cell lines and cannot easily be applied to fresh peripheral blood lymphocytes. Despite this, a model that has gained popularity is that a completely quiescent T cell may contain a fully integrated provirus, in a true state of latency with no active transcription. This cell must have a history of transient activation in order to contain integrated viral DNA (see chapter 6). The cell is then activated by antigen with co - stimulation *or* by cytokines such as TNF. HIV is then transcribed and a series of virally mediated positive feedback mechanisms maintain transcription, such as Tat transactivation, HIV induced NFκB p105 transcription and proteolysis and induction of a TNF autocrine loop.

The TNF autocrine loop is the reciprocal maintenance of HIV and TNF transcription - each mutually inducing one another as described above. For this to occur in an autocrine fashion, cells must be in an activated state and indeed this phenomenon has not been described in resting cells. If these transcriptional regulatory mechanisms maintain HIV replication, their blockade could prove useful therapeutically, without impeding important intracellular signals.

The TNF autocrine loop has been suggested as having a particular role in CD45RO+ CD4+ cells, which carry most HIV DNA *in vivo*<sup>221</sup> and secrete more TNF following stimulation<sup>222-224</sup>, although little is known about differences in TNF mediated signal transduction in these cells. This proposal has been supported by the finding that CD45RO+ cells produce more p24 and SF after addition of TNF or mitogen, an effect abrogated by anti - TNF, following *in vitro*<sup>223-225</sup> or *in vivo* infection<sup>226</sup>.

In this chapter the effects of TNF on CD45RA+ and RO+ CD4+ lymphocytes is studied with the primary aim of evaluating signal transduction in the putative TNF autocrine loop. A secondary aim was to evaluate the effects of raised levels of TNF, as seen in HIV infection, on these two populations of cells.

## 7.2 Results.

### 7.2.1. NFκB Induction By TNF In CD45RA+ And CD45RO+ CD4+ Cells.

Separated cells were prepared under conditions of minimal *in vitro* stimulation; male human serum was used throughout and sera and antibodies were passed through a polymyxin B (Sigma) column<sup>227</sup> to attain LPS concentrations below 0.1 U/ml, as detected by a Limulus Amoebocyte assay (E-Toxate, Sigma). Since monocytes can release TNF on adhesion, they were removed as rapidly as possible in sequential 30 minute adhesion steps. Separated cells were stimulated with immobilised anti - CD3, PMA 5ng/ml or TNF 1000u/ml for 8 hours.

CD45RO+ cells contain more protein than RA+ cells<sup>228</sup>. In band shift assays nuclear protein extract from  $5 \times 10^5$  cells of either population was used in each lane, rather than a fixed amount of protein.

The basis of the electro - mobility - shift - assay is that after mixing nuclear protein extract and oligonucleotide, proteins binding DNA motifs will impede their progress through a polyacrylamide gel resulting in the formation of bands. In lanes 1 and 2 of Figure 7.2A the recombinant protein KBF1, kindly provided by Dr Ron Hay, was used instead of cell extract. This protein has homology to NFκB p50<sup>229</sup> and could be seen here to bind the HIV tandem κB motif, but not an oligonucleotide in which the GGG/CCC sequences in each κB motif were altered to CTC/GAG (see figure 2.1), hence confirming the specificity of the oligonucleotides. Nuclear extract from HeLa cells (Lane 3) produces 2 bands of slower mobility than KBF1. Since the KBF1 sequence was originally derived from a HeLa protein<sup>229</sup>, the slower bands in lane 3 may represent (p50)(p65) dimers<sup>230</sup> or the HeLa p57 κB binding protein<sup>231, 232</sup>.

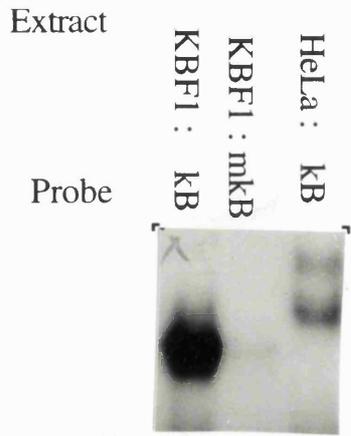
In Figure 7.2.B the specificity of the oligonucleotides and binding procedure was evaluated using nuclear protein extract from enriched CD4+ T cells (prepared by using the first step in the immunomagnetic separation only; see Section 2.2.2.) stimulated with immobilised anti - CD3. In the first lane, with κB as the binding oligonucleotide, 3 bands can be discerned; when mutant κB (mκB) is used the fastest is diminished considerably (lane 2). In lane 3, a 50 fold excess of unlabelled κB oligonucleotide was added in addition to κB; this can be seen to diminish the intensity of the fast band only. A 50 fold excess of unlabelled mutant κB oligonucleotide had no negative effects on the fast band (lane 4). These data suggest that, in this experimental set up, phorbol ester activation produces a number of κB binding proteins; only the faster migrating protein binds the κB oligonucleotide in preference to mκB and thus has the functional properties of an NFκB family member, as noted by others<sup>74</sup>.

Figure 7.2.C shows a band shift assay conducted on separated CD45RA+ and RO+ CD4+ T cells. In both populations untreated cells and cells treated with TNF show the same pattern of no detectable κB binding. Cells from either population treated with

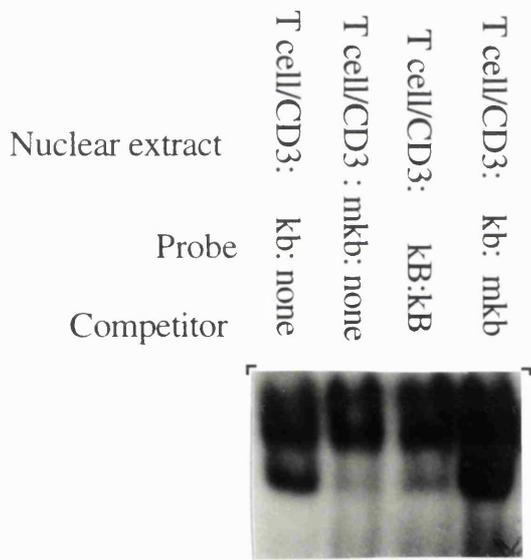
immobilised anti - CD3 show the induction of a faster migrating protein and loss of the higher molecular weight complexes. Since resting peripheral blood T cells cannot support HIV replication<sup>233-235</sup>, it is likely that the bands in lanes 1, 3, 4 and 6 represent constitutive factors with no transcriptional activity, similar to the high molecular weight  $\kappa$ B binding proteins in Figure 7.2 A and B. Since immobilised anti - CD3 transactivates HIV in the absence of accessory cells by inducing NF $\kappa$ B<sup>37, 39, 53,236</sup>, the inducible bands in lanes 2 and 5 of figure 7.2C represent functional NF $\kappa$ B. Hence TNF does not appear to be able to induce NF $\kappa$ B in resting T cells expressing either CD45RA or CD45RO.

# Figure 7.2. NFκB Binding In CD45RA+ And CD45RO+ CD4+ T Cells.

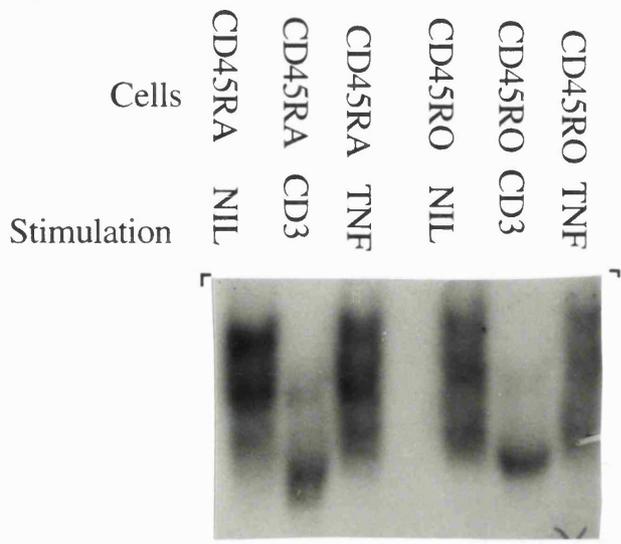
A: Protein binding to kB and mutant kB oligonucleotides.



B: T cell extract binding to kB and mutant kB oligonucleotides.



C: Binding of nuclear extract from CD45RA/RO cells before and after stimulation.



### 7.2.1. Redox Changes With TNF In CD45RA+ And CD45RO+ CD4+ Lymphocytes.

The above data show that TNF does not induce NFκB in these resting T cell populations. PMA and, particularly, TNF induce NFκB by increasing the intracellular ROI concentration<sup>54</sup>; hence, ROIs provide a useful way of determining whether more proximal signalling events take place after exposure of these populations to TNF.

The effects of TNF exposure on intracellular ROIs was measured indirectly, by exposing cells to stimuli and then loading them with monochlorobimane (MCB) 120mM. MCB fluorescence increases linearly with concentrations of glutathione (GSH) and time; the slope of emission at 420nm over time is related to the intracellular glutathione concentration and inversely to ROI concentration.

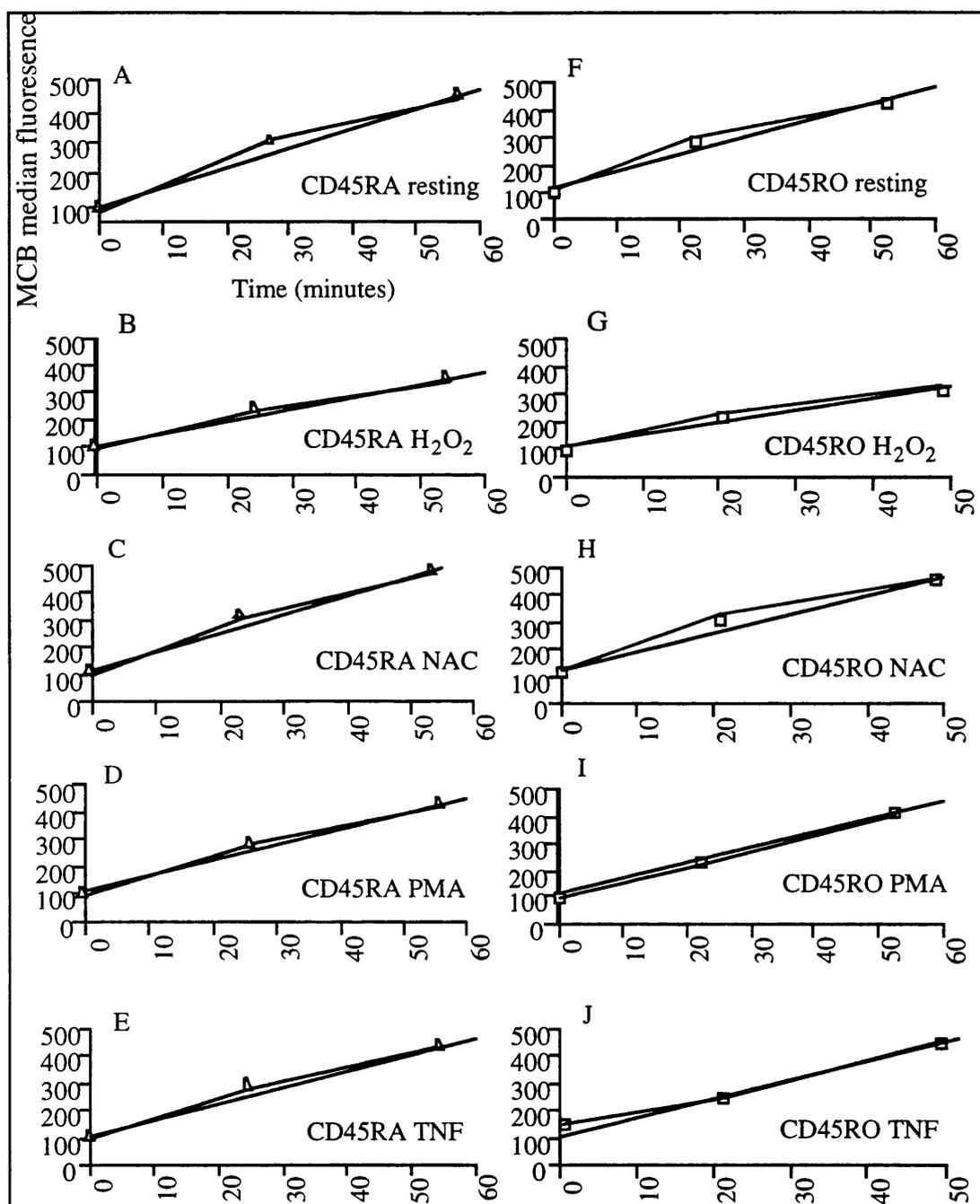
Table 7.2. Relative Intracellular GSH Concentrations.

	Resting	H <sub>2</sub> O <sub>2</sub> (20nM)	NAC (50nM)	PMA (5ng/ml)	TNF (1000U/ml)
CD45RA+	6.15 (1.00)	4.61 (0.75)	6.98 (1.13)	5.61 (0.91)	6.18 (1.00)
CD45RO+	6.14 (1.00)	4.53 (0.74)	6.86 (1.17)	5.73 (0.93)	6.25 (1.02)

The graphs in Figure 7.3 represent increases in emission at 420nm over time for CD45RA+ (A-E) and CD45RO+ cells (F-J) with no stimulation (A, F), hydrogen peroxide (B, G), N acetyl cysteine (NAC) (C, H), PMA (D, I) and TNF (E, J). The numbers in Table 7.2 are the slopes of the regression lines and values relative to untreated cells, which have an established role in evaluating changes in GSH in T cell lines<sup>54, 237</sup>.

Without stimulation both populations contain comparable levels of GSH. Hydrogen peroxide and NAC were added to determine the efficacy of the assay in detecting changes in intracellular GSH, since these agents deplete and replenish sulphhydryl residues, respectively. The addition of hydrogen peroxide causes a clear decrease in GSH whilst NAC causes a small increase in GSH concentration, as previously noted in T cell lines<sup>203</sup> and confirming the validity of the assay. PMA stimulation results in a fall in the intracellular GSH concentration comparable with changes noted in PMA treated T cell lines<sup>54</sup>; the fall was similar in CD45RA+ and RO+ cells and confirms that these cells are capable of changing their redox state on appropriate stimulation. The finding that both populations contain similar amounts of GSH at rest and after oxidative/reductive stress is interesting since GSH content is related to proliferative capacity of lymphocytes<sup>202</sup> and CD4+ cells with high GSH content are lost early in HIV infection<sup>196,198</sup>, findings which may have suggested higher GSH in resting CD45RO+ cells.

Figure 7.3. Monochlorobimane Fluorescence In CD45RA+ And CD45RO+ CD4+ Cells.



In a variety of cells, including T cell lines, TNF induces ROIs and depletes GSH<sup>203,204,238</sup>. However, TNF had no effects on the redox state in these resting populations of T cells.

The processes that follow TNF binding to its receptors and precede the increase in intracellular ROI concentration (and PKC activation) are unknown. TNFR expression on these quiescent CD4+ T cells was thus determined to assess whether these could account for the inability to induce NFκB and ROIs after exposure to TNF.

### 7.2.3. TNF Receptor Expression On CD45RA+ And CD45RO+ CD4+ T Lymphocytes.

Peripheral blood lymphocytes were separated into CD45RA+ and CD45RO+ CD4+ lymphocytes, as described in Chapter 2. MHC Class II antibody L243 was included in the cocktail of mAbs used and so T cells activated *in vivo* or in the early stages of separation were excluded. Details of stimulation and staining are given in section 2.3.5.

**Table 7.3. Expression Of TNF Receptors By Resting T Cells.**

	CD4+ CD45RA+	CD4+ CD45RO+
Irrelevant FITC conjugated antibody	77 (11)	82 (13)
IL2R $\alpha$	96 (8)	138 (27)
MHC Class II	74 (8)	79 (11)
Irrelevant biotinylated antibody.	85 (11)	89 (13)
TNF receptor p75	98 (14)	126 (29)
TNF receptor p55	89 (27)	88 (21)

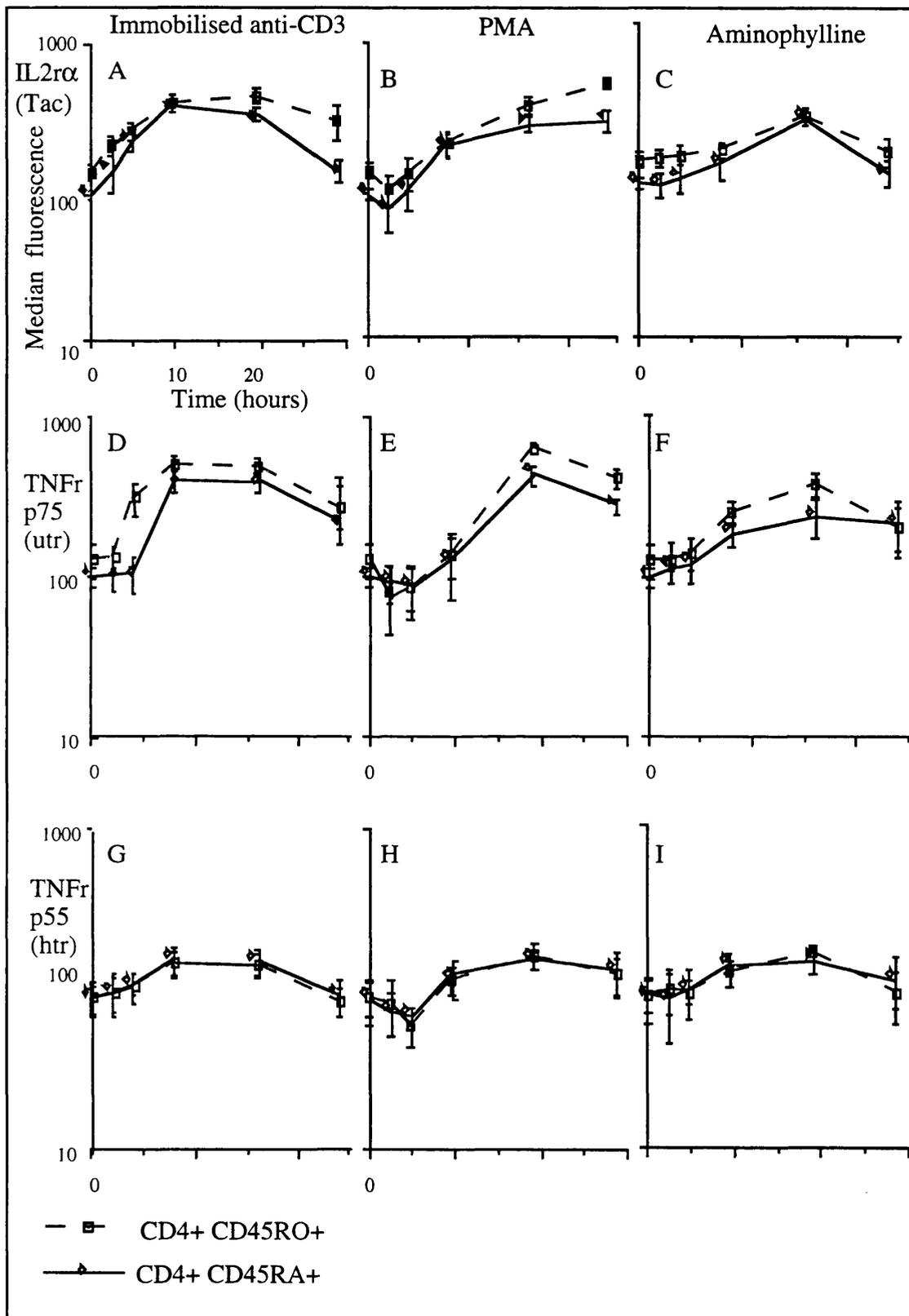
Median fluorescence (arbitrary units) and (sd) from 11 experiments.

In table 7.3 it can be seen that MHC Class II positive cells are excluded during separation, although IL2R $\alpha$  is homogeneously expressed, particularly on CD45RO+ cells as previously noted<sup>239</sup>. (The observation that after exclusion of Class II positive cells some IL2R $\alpha$ + cells remain is unexplained and was also noted during the preparation of CD45RO+ Class II+ and CD45RO+ Class II- cells for the experiments described in chapter 6; the data in Table 6.1 give different values for median fluorescence because of lower FL1 amplifications used in these experiments).

TNFR p55 (reactivity with mAb htr) was barely detectable above background, in both populations. Both populations express detectable TNFR p75 (utr) although this receptor is present more abundantly on CD45RO+ cells. On stimulation, all cells upregulated TNFR homogeneously, suggesting expression of these receptors did not define specific subsets of cells.

To assess how expression of the TNFR change after stimulation (figure 7.3), separated cells were exposed to immobilised anti - CD3 (A,D,G), aminophylline 1mM (B,E,H) or PMA 5ng/ml (C,F,I) for various intervals. Cells were then stained for IL2R $\alpha$  (A(B,C), TNFR p75 (D,E,F) and TNFR p55 (G,H,I,) expression. The mean of median fluorescence for each time point and standard deviation for 4 experiments are shown in figure 7.3.

Figure 7.4 Expression Of IL2R $\alpha$ , TNFR p75 And p55 On CD45RA+ And CD45RO+ CD4+ Cells.



Stimulation with immobilised anti - CD3, PMA and the phospho - di - esterase inhibitor aminophylline up - regulated IL2R $\alpha$  in both populations; these stimuli induce NF $\kappa$ B, via PKA in the case of aminophylline<sup>57</sup>. PMA induced an initial down -

regulation of IL2R $\alpha$ . Up - regulation of IL2R $\alpha$  was more marked in CD45RO+ cells after PMA, aminophylline and, as previously described<sup>240</sup>, with anti - CD3.

The two TNFR were up - regulated differentially, as noted before in other cell types<sup>241-243</sup>. All three stimuli up - regulated TNFR p75 more in CD45RO+. PMA initially down - regulated p75, as previously noted<sup>242, 244, 245</sup>. TNFR p55 was much less up - regulated than p75 with all of the three stimuli and affected both CD45RA+ and RO+ cells equally. Notably, p55 expression was down regulated after PMA treatment, suggesting that fresh T cells do express small quantities of this receptor. These findings are reminiscent of the observation that the related molecule Fas is expressed at low level on CD45RO+ cells and negligibly on CD45RA+ cells; both populations upregulate Fas in conditions of activation<sup>315</sup>.

The up - regulation of these receptors was largely sensitive to cycloheximide (not shown) and hence is mediated by *de novo* protein synthesis.

#### 7.2.4 The Effects Of TNF Co - Stimulation On Sub - Optimally Stimulated CD45RA+ And CD45RO+ CD4+ Cells.

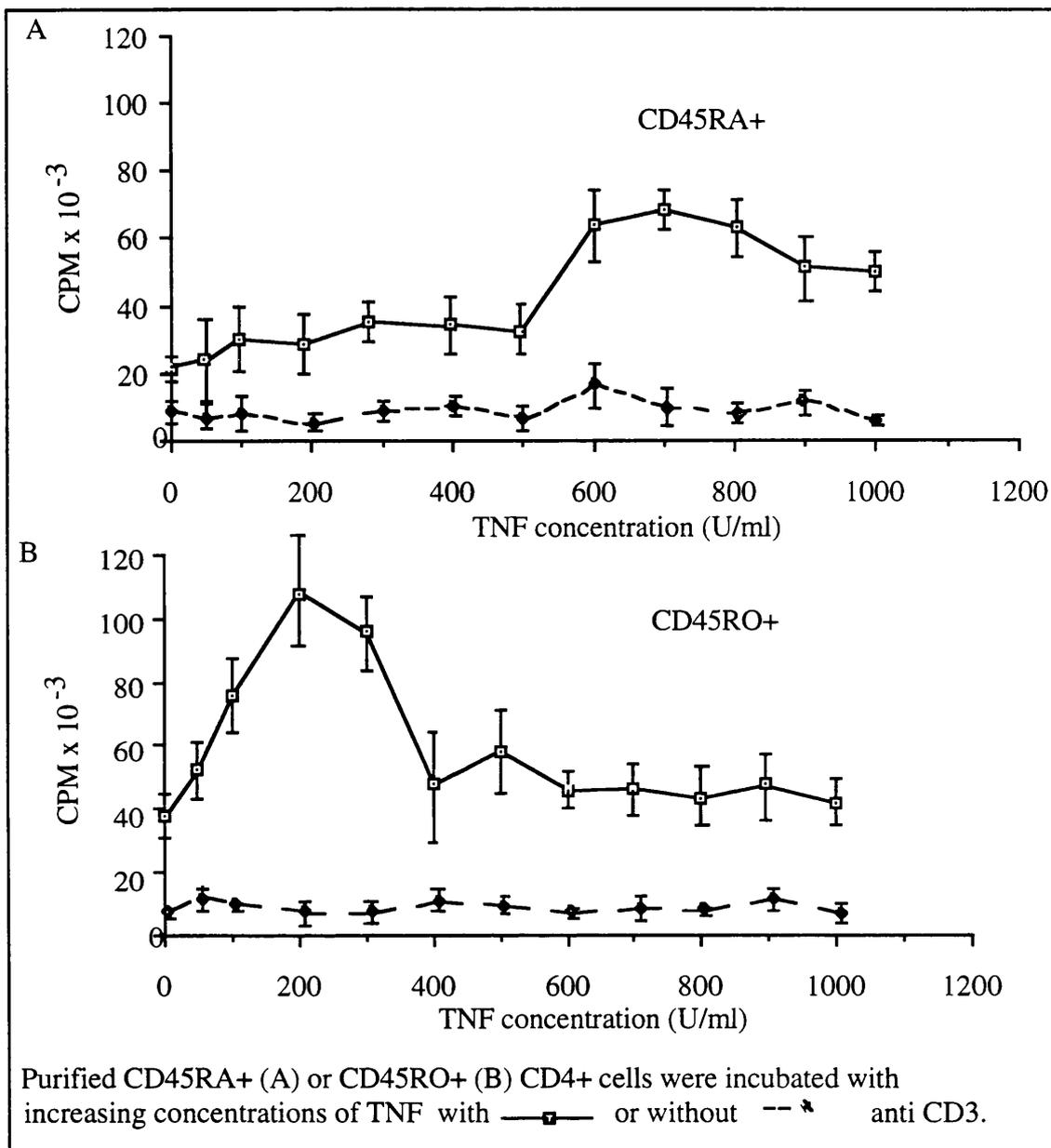
These data show that TNFR expression in unstimulated cells is minimal but is up - regulated after stimulation. It remains possible that on stimulation CD45RA+ and RO+ CD4+ cells respond differently to TNF. Because stimulated T cells express NF $\kappa$ B it would not be easy to detect changes in this transcription factor after TNF co - stimulation without using reporter genes. Instead differences in the co - stimulatory effects of TNF on sub - optimally stimulated cells were studied.

In preliminary experiments, the CD3 antibody UCHT1<sup>246</sup> was titrated by coating cells with different concentrations of UCHT1, placing them on rabbit - anti - mouse immunoglobulin, immobilised on flat bottom 96 well plates in alkali conditions, culturing for 4 days, pulsing with tritiated thymidine and then harvesting. A concentration of UCHT1 of 5 $\mu$ g/ml was found to give approximately 50% optimal stimulation (not shown) and this was used in subsequent experiments.

Figure 7.4, representing data typical of 6 experiments, shows that after 4 days incubation there is minimal proliferation in T cells of either population in the absence of anti - CD3, even when high concentrations of TNF are present. In the presence of immobilised anti - CD3, both populations proliferate. As previously noted<sup>247,248</sup> CD45RO+ cells proliferate more than CD45RA+ cells at suboptimal concentrations of immobilised anti - CD3 in the absence of TNF (in these experiments CD45RA+ 22640cpm (sd 3760): CD45RO+ 36850cpm (sd 8120)). Addition of low concentrations of TNF to CD45RO+ cells augments proliferation up to threefold at concentrations of 200U/ml; at higher concentrations of TNF this co - stimulatory effect is lost. CD45RA+ cells behave differently; TNF at concentrations up to approximately 500U/ml has no effects on proliferation of CD45RA+ cells; subsequently there is a modest augmentation of proliferation up to concentrations of 2,000U/ml.



Figure 7.4. Effects Of TNF And Anti - CD3 On Proliferation Of CD45RA+ And CD45RO+ CD4+ Cells.



To exclude a kinetic difference between the two populations, cells were incubated for different periods before pulsing with tritiated thymidine. At this concentration of immobilised anti - CD3 proliferation was minimal at 3 days in both populations (not shown). At days 5 and 6, cells stimulated with TNF alone showed only minimal proliferation. Figure 7.5 shows the effects of TNF on anti - CD3 stimulated cells at 5 and 6 days. Proliferative response decreases at these times, but the pattern of enhanced CD45RO+ cell proliferation at TNF concentrations between 200 and 500 U/ml and enhanced CD45RA+ cell proliferation at concentrations above 500U/ml persists.

TNF mediates its effects on proliferation by upregulating IL2R $\alpha$ <sup>249-252</sup> without affecting IL2 secretion<sup>250, 252</sup>. To assess the effects of TNF on IL2R $\alpha$  in CD45RA+ and RO+ cells, resting and anti - CD3 stimulated populations were incubated with varying doses of TNF for 12 hours and then stained with FITC conjugated anti - CD25. In

Figure 7.6 it can be seen that TNF at 1000U/ml has no effects on IL2R $\alpha$  expression in either population without anti - CD3. In cells treated with immobilised anti - CD3, IL2R $\alpha$  expression is up - regulated. Addition of TNF, concurrently with anti - CD3 stimulation leads to a further increase in IL2R $\alpha$  expression; in CD45RO+ cells addition of TNF reaches its full effect at 200U/ml whilst in CD45RA+ cells TNF begins to augment IL2R $\alpha$  expression between 200 and 500U/ml. Hence the co - stimulatory effects of TNF and the higher concentrations requirements of CD45RA+ cells may be mediated by IL2R $\alpha$  expression. In other experiments rIL2 was added at 50U/ml to separated cells being stimulated with anti - CD3 and TNF (not shown). IL2 increased proliferation in anti - CD3 stimulated cells but did not affect the dose related co - stimulatory effects of TNF or the decrease in proliferation of CD45RO+ cells at higher TNF concentrations.

Figure 7.5. Proliferation Of CD45RA+ And CD45RO+ CD4+ Cells In Response To Anti - CD3 And TNF At 5 And 6 Days.

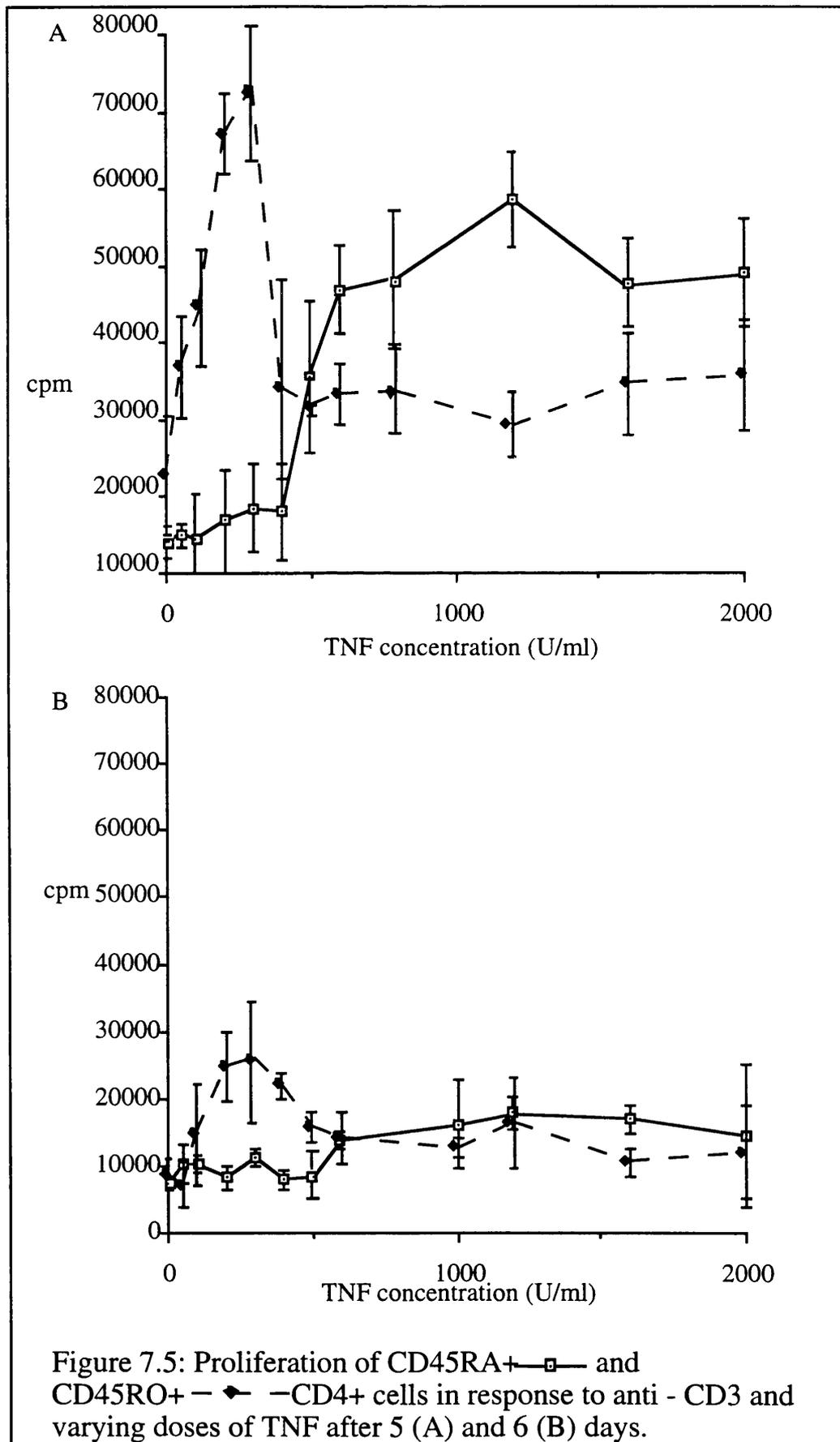
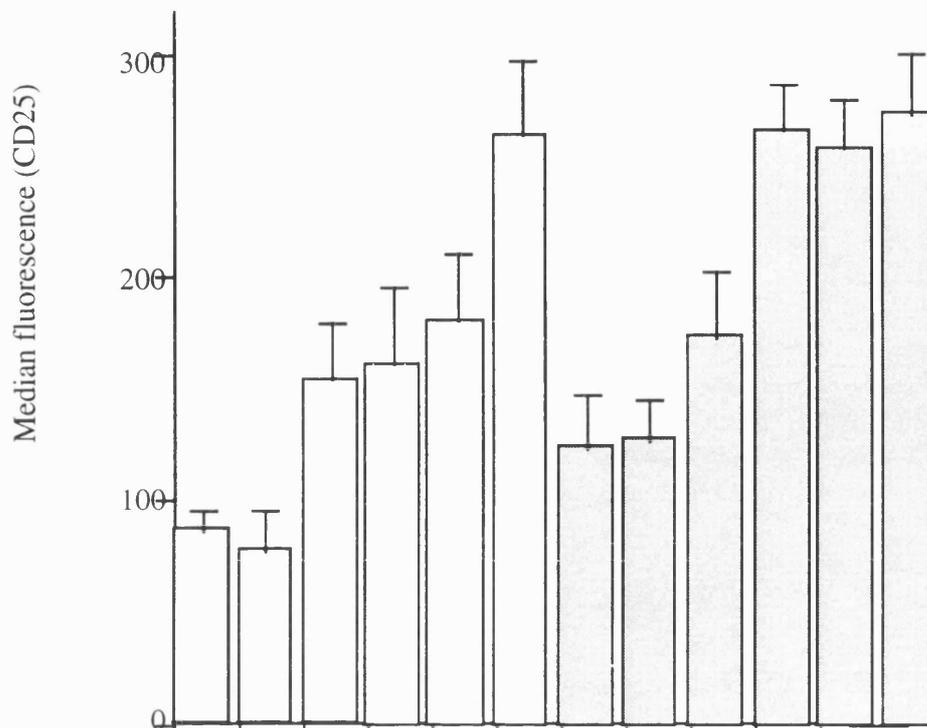




Figure 7.6. Effects Of TNF On IL2R $\alpha$  Expression In CD45RA+ And CD45RO+ Cells.



Cells	CD45RA+						CD45RO+					
Anti CD3	-		+				-		+			
TNF U/ml	0	1000	0	200	500	1000	0	1000	0	200	500	1000

One possible explanation for the decreased proliferation of CD45RO+ cells is that they undergo apoptosis when co - stimulated with high doses of TNF. Apoptosis occurs predominantly in cells expressing this CD45RO isoform in the periphery<sup>253-255</sup> and perhaps also in the thymus<sup>256</sup>.

Figure 7.7 shows the result of an experiment in which DNA was electrophoresed to assess the degree of DNA fragmentation after stimulation with anti - CD3 and TNF. A T cell clone (kindly provided by S. Schneider, Deutschesrheumaforschungszentrum) deprived of IL2 overnight<sup>257</sup> was included as a positive control. DNA fragmentation is clearly seen as a series of “ladders”, multimers of 200 base pair DNA fragments, characteristic of apoptosis<sup>258</sup>. After 48 hours stimulation with anti - CD3 with or without TNF does not result in detectable DNA fragmentation in either the CD45RA+ or CD45RO+ cell population.

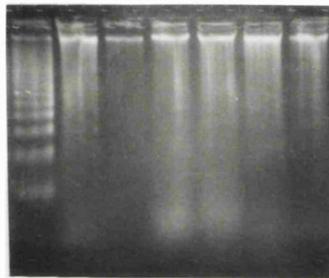
Although the positive control gave clear results in the DNA electrophoresis assay, the sensitivity may not have been high enough to detect apoptosis in a sub - population of dividing CD45RO+ cells. As auxiliary tests of apoptosis and to look for apoptosis in small sub - populations of cells, flow cytometry was performed to assess

changes in cell size or granularity<sup>259, 260</sup> and changes in ethidium - mono - azide staining<sup>261</sup>. The presence of TNF did not alter these parameters (not shown).

Finally, IL2 has been shown to rescue CD45RO+ cells from apoptosis<sup>253,254,260</sup>, but made no impact on CD45RO+ cell proliferation at high TNF concentrations (see above), suggesting apoptosis is not involved in the reduced proliferation of CD45RO+ cells at high concentrations of TNF.

### Figure 7.7. No Evidence Of DNA Fragmentation In CD45RA+ And CD45RO+ Cells Stimulated With TNF And Anti - CD3.

Cells	C	CD45RA+			CD45RO+		
		-	+	+	-	+	+
Anti - CD3	O	-	+	+	-	+	+
TNF 1000U/ml	N	-	-	+	-	-	+



### 7.3. Discussion.

The data in this chapter show that NFκB is not active in resting CD4+ T cells and cannot be induced by TNF alone, probably because of inadequate expression of TNF receptors. Similarly changes in redox potential, IL2Rα expression or proliferation are not induced in resting cells by TNF. TNF receptor expression is up - regulated after T cell stimulation and then mediates TNF co - stimulation of proliferation and IL2Rα upregulation. These effects occur at lower concentrations of TNF in CD45RO+ cells, although in this population the co - stimulatory effects are lost with high concentrations of TNF, through a mechanism which appears to be independent of apoptosis.

### Models Of TNF Mediated Transcriptional Changes And The TNF Autocrine Loop.

Experiments on the effects of TNF on unstimulated CD45RA+ and RO+ T cells latently infected with HIV are difficult to do, since a degree of activation is a prerequisite for *in vitro* infection and with CD45RA+ cells may result in acquisition of the CD45RO+ phenotype. In the CD45RO+ population activation sufficient to up - regulate IL2Rα expression is required to optimise infection; IL2Rα expression parallels

TNFR expression and so a period of resting would be required before the effects of TNF on *bona fide* quiescent cells could be studied.

Studies on *in vivo* HIV infection show integrated DNA to be present largely in cells expressing activation markers IL2R $\alpha$ <sup>262</sup> or MHC Class II<sup>263</sup>. Elimination of IL2R $\alpha$ + cells with ricin linked anti - Tac results in decreased p24 production by *in vivo* infected CD4+ lymphocytes; because p24 production can be rescued by stimulation after IL2R $\alpha$ + cells have been eliminated<sup>262</sup>, a small population of infected truly quiescent cells does exist. These cells would be optimal material for conducting experiments on the significance of the TNF autocrine loop.

Enhanced TNF secretion by HIV infected lymphocytes and monocytes coupled to TNF mediated induction of NF $\kappa$ B and transactivation of HIV have been postulated as an autocrine loop capable of 1)breaking viral latency and 2)maintaining HIV replication<sup>72,148,154,210,264,265</sup>. The autocrine loop requires both TNF secretion and the presence of TNF receptors. From this model has emerged the idea that breaking the loop may be useful therapeutically and an explanation of why HIV replication is dominant in CD45RO+ CD4+ cells<sup>223</sup>.

The data in this chapter are limited by the two provisos affecting *in vitro* and *in vivo* infection, as discussed in the introduction. However, the data presented in this chapter suggest that TNF may not have a primary role in breaking viral latency or maintaining replication in T cells. Firstly, in quiescent T cells paucity of TNFR expression prevents transmission of signals from exogenous TNF. Although HIV up - regulates TNFR expression in T cell lines<sup>181</sup>, this does not seem to be the case in fresh T cells<sup>224</sup>. The very stimuli that up - regulate TNFR expression also induce optimal HIV transcription<sup>53</sup>, hence bypassing the need for TNF in breaking viral latency.

If the TNF autocrine loop cannot induce HIV transcription in resting T cells, how important is it in the maintenance of HIV replication in recently activated cells? After polyclonal activation primary T cells secrete TNF for about 5 days<sup>222,266</sup>. The data presented here show that up - regulation of TNFR is even more evanescent. It has been suggested that TNF secretion and TNFR expression may be induced by HIV infection. That TNF secretion is a consequence of HIV infection of T cells remains unclear (see section 7.1.6) and since there is no confirmed evidence showing that HIV up - regulates TNFR, the idea that HIV supports the TNF autocrine loop long after T cell stimulation remains unsupported. Another possible explanation for sustained HIV replication after T cell stimulation is that once HIV transcription is temporarily increased, HIV gene products, such as gp120 and Tat, maintain activation<sup>30, 267-276</sup>.

The data presented here do not determine whether TNF increases the amount of NF $\kappa$ B induced after anti - CD3 mediated activation. The electro - mobility - shift assay is unable to detect this as it is a qualitative assay; once a band has been induced it is not possible to detect further induction, unless of a different mobility.

Studies using HIV - LTR - reporter gene constructs or native gene transcription are required to determine super induction by TNF in activated cells. That TNF induces an additional signal after anti - CD3 treatment is strongly inferred by the data showing

TNF further stimulates anti - CD3 mediated proliferation and extends previous observations<sup>252</sup>. This enhancement of proliferation was mediated, at least in part, by up - regulation of IL2R $\alpha$ , although the second messenger system used was not determined.

### 7.3.2. TNF And Anti - HIV Therapies.

Because TNF has been thought to have a pivotal role in HIV pathogenesis<sup>207</sup>, attempts have been made to exploit therapeutically the hypothesis that TNF breaks latency and/or maintains replication. Oxpentifylline inhibits TNF secretion<sup>277</sup> and HIV replication in acutely but not chronically infected Jurkat cells stimulated by supernatant from LPS stimulated macrophages<sup>278</sup>, but *in vivo* oxpentifylline shows no clear reduction in virus load on its own, although it may potentiate zidovudine<sup>279</sup>. Thalidomide also reduces TNF secretion and HIV replication in *in vitro* systems<sup>280</sup>; results of *in vivo* studies are awaited.

Recombinant soluble (sTNFR) has been synthesised<sup>281</sup> and shown to be effective in *in vivo* animal models of inflammation<sup>282</sup>. sTNFR inhibits HIV replication in a T cell line treated with TNF<sup>283</sup>, but it remains unclear whether these agents reduce TNF availability in the long term, rather than buffer acute increases in TNF concentration with sustained levels over several days<sup>284</sup>. Furthermore, endogenous sTNFR levels are already raised in HIV infection<sup>167, 182</sup>. Sulfasalazine inhibits TNF binding to TNFR<sup>285</sup>, but has not been investigated in HIV infection.

ROI induction by TNF can be inhibited *in vitro* by anti - oxidants such as NAC<sup>54,203,204</sup> and ascorbic acid<sup>286</sup>, with a commensurate reduction in HIV transcription. Although there are no clinical data showing anti - HIV efficacy of anti - oxidants, these agents have also been proposed as having an immunostimulatory effect<sup>200</sup>. NF $\kappa$ B induction by TNF has recently been shown to be inhibited by aspirin; *in vitro*, aspirin also HIV - reporter gene transcription; clinical studies of this agent are underway<sup>312</sup>.

The corollary of these data is that administration of TNF to patients with AIDS related complex showed no increase in p24 or isolation rate of HIV from blood<sup>287</sup>.

Although simple *in vitro* studies show efficacy of some anti - TNF agents, when *in vivo* data is available it suggests that either the TNF autocrine loop is not important *in vivo* or that it is not easily interrupted. Exploration of other *in vivo* models, such as cats infected with the feline immunodeficiency virus (which also requires transactivation through NF $\kappa$ B<sup>288</sup>), may help define the roles of these agents in HIV infection and inflammation.

### 7.3.3. TNF And HIV In Cells Expressing CD45RA And CD45RO.

CD45RO+ cells secrete more TNF<sup>222,223</sup> and other cytokines than their CD45RA+ counterparts, although this is not a consistent finding<sup>289</sup>. It appears that

signals through CD45 itself may modulate secretion of this cytokine<sup>290</sup> along with other aspects of activation. The TNF autocrine loop has been invoked as an explanation of why HIV replicates preferentially in CD45RO+ CD4+ lymphocytes<sup>223</sup>.

In one set of experiments, a complex system determined SF and low (picogram) levels of p24 production in mixtures of the HIV permissive T cell line C8166 and separated CD45RA+ and RO+ cells after *in vitro* HIV infection<sup>223</sup>. p24 production and SF occurred predominantly in the mixtures containing CD45RO+ cells and SF was abrogated by the addition of anti - TNF. These data are confounded by MHC Class II/IL2R $\alpha$ + cells not being excluded from the CD45RO+ population and containing a sub set with extreme vulnerability to HIV. and hence be more vulnerable to HIV infection. In these conditions TNF may be secreted in response to allogeneic stimulation<sup>291</sup> and not a direct consequence of HIV infection. Finally, addition of anti - TNF reduces ICAM - 1 expression<sup>292</sup> and may thus abrogate SF. These data do not support the existence of a more effective TNF autocrine loop in the CD45RO+ population.

Similar experiments on CD45RA+ and RO+ cells infected *in vivo* show preferential replication (p24 production) in PHA stimulated CD45RO+ cells from patients with asymptomatic HIV infection<sup>226,289</sup>; addition of PMA and TNF increased replication in CD45RO+ cells only<sup>226</sup>. In these experiments, MHC Class II+ or IL2R $\alpha$ + cells were also not removed and so a greater carriage of HIV provirus could be expected in CD45O+ cells<sup>262, 263</sup>; activation by any means would then be expected to produce more p24 in these cells. Enhanced replication in these experiments may be consequence of carriage rather than increased sensitivity to TNF.

#### 7.3.4. TNF And Proliferation In Cells Expressing CD45RA And CD45RO.

TNF has co - stimulatory activity in T cells<sup>250,252,293,294</sup> and thymocytes<sup>295</sup>, part of which is dependent on IL1 secretion by TNF stimulated monocytes<sup>296</sup>, but most is exerted directly on T cells<sup>252,266,294,295</sup>. In stimulated T cells, TNF up - regulates IL2R $\alpha$  expression<sup>249,297,298</sup>, explaining why TNF mediated enhancement of proliferation occurs most when concentrations of IL2 are limiting<sup>250,299</sup>. Other workers have found that co - stimulation mediated by TNF occurs just as well when IL2 concentrations are saturating<sup>294</sup> and have instead noted that co - stimulation is optimised when cell concentrations are limiting<sup>294</sup>, leading to the suggestion that TNF co - stimulates by providing cell - cell communication<sup>294</sup> either as TNF TNFR ligand pairs (analogous to CD40 - CD40L<sup>300</sup>) or by upregulating adhesion molecules<sup>292</sup>.

The co - stimulatory effects of TNF are generally only observed when TNF is present early in activation<sup>252,294</sup> and in many experimental systems<sup>250,252,294</sup> a dose response curve is seen, which reaches a plateau at between 100 and 1000u/ml. In other systems<sup>291,299,301</sup> the co - stimulatory effects of TNF are lost at high concentration, although this has not been explained.

Although CD45RO+ cells are known to secrete more TNF than their RA+ counterparts<sup>222</sup>, but little is known about the responses of these populations to TNF. Unutmaz has shown that resting CD4+ lymphocytes, all negative for IL2R $\alpha$  or MHC Class II expression, proliferate in response to cytokines without the need for TcR stimulation<sup>115</sup>. CD45RO+ respond to the combination of IL2 and TNF, whilst CD45RA+ cells are fastidious and require the addition of IL6. IL6 has a special role in co - stimulating proliferation in CD45RA+ cells<sup>302</sup>, which do not secrete this cytokine<sup>302, 303</sup> and rely on IL6 secretion by CD45RO+ cells<sup>304</sup>. In cells able to secrete IL6, secretion can be up - regulated after exposure to TNF<sup>56, 297</sup>, although IL6 does not induce TNFR<sup>242</sup>. A special characteristic of the type of proliferation in the CD45RA+ cells was that CD45RO expression did not appear to be induced<sup>115</sup>; IL6 has previously been shown to up - regulate CD45RA on CD45RA+ T cell clones<sup>305</sup>.

The data presented here and that of Unutmaz agree that TNF alone has no effects on resting CD4+ lymphocytes and that CD45RA+ and RO+ cells respond differently to TNF as a co - stimulus; notably, at concentrations of approximately 500U/ml TNF has different effects on CD45RA+ and RO+ cells. In the data presented in this chapter, signalling through CD3 is required to elicit TNF co - stimulatory effects, but in Unutmaz's experiments, addition of IL2 suffices.

In the model that emerges, CD45RO+ cells express low levels of IL2R $\alpha$  and TNFR p75 at rest. On activation by anti - CD3 both these receptors are rapidly up - regulated. IL2 enhances TNFR expression<sup>242</sup> and TNF up - regulates IL2R $\alpha$ . Hence a positive feedback operates early and even at low concentrations of exogenous TNF; this may be augmented by the ability of CD45RO+ cells to secrete IL6. CD45RA+ cells are disadvantaged because the baseline levels of TNFR p75 and IL2R $\alpha$  are lower, they up - regulate TNFR p75 slightly later, at a time when it may be less effective. When TNF is added, CD45RA+ cells require higher concentrations for co - stimulation because the amplification provided by IL6 and cytokine receptor expression is diminished in this population. Thus CD45RO+ cells are hyper - reactive in this system<sup>306</sup>.

Inhibition of proliferation at high concentrations of TNF, as occurs in the CD45RO+ population in this study, has been noted in other types of cells<sup>291, 299, 301</sup> and could be explained by apoptosis. IL2 starvation<sup>257</sup> or priming<sup>307</sup> would be unlikely to cause this effect, since it occurred whether or not IL2 was saturating. At the higher concentrations of TNF at which proliferation inhibition occurred, the lower affinity TNFR p55 could be active and is known to be able to induce apoptosis<sup>308</sup>.

Activation associated lymphoid death (AALD) differs from apoptosis in that primed cells, expressing high levels of IL2R $\alpha$ , cannot be rescued by addition of IL2<sup>254</sup>. This is similar to the phenomenon noted here in CD45RO+ CD4+ cells treated with doses of TNF and has been proposed as an explanation for the failure of CD4+ cells to respond to anti - CD3 in early HIV infection<sup>309</sup>.

Although apoptosis and AALD were plausible mechanisms for inhibition of proliferation of CD45RO+ cells at high TNF concentrations, no evidence of these processes was detected in this study. It remains possible that only a very small

percentage of cells over - stimulated in response to TNF undergo apoptosis. For example, Unutmaz found only 1 in 33 CD45RO+ cells respond to the combination of IL2, IL6 and TNF<sup>115</sup>, but even sensitive assays failed to detect death in the CD45RO+ population in this study.

Alternatively, a third TNFR with very low affinity may come into play and deliver a negative signal. TNFRp<sup>310</sup> may be a candidate for this although its affinity for TNF is not defined. The molecular basis for anti - growth without death is unclear, although activation through TNFRp or p55 may switch on a gene with capability to inhibit cells cycling, such as p34<sup>cdc2311</sup>.

In summary, TNF alone exerts no detectable effects on resting T cells and the breaking of viral latency cannot be attributed to it. In co - stimulation TNF exerts pleiomorphic effects on CD45RA+ and CD45RO+ cells, in part reflecting hyper - reactivity and in part reflecting undefined pathways.

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# Chapter 8. Conclusions.

## Review Of Data Presented.

The data presented in this thesis show that HIV and its component proteins may interact differently with CD4+ T cells expressing either CD45RA or CD45RO. Both populations of cells express similar numbers of CD4 molecules, and all the domains required for HIV infection are fully expressed. CD45RA+ cells have a slightly higher affinity for gp120. Following binding, gp120 is lost from the surface of CD45RA+ cells slightly more rapidly, possibly because on these cells stable complexes form less readily. This fits with the finding that gp120 induces a somewhat greater magnitude calcium influx in CD45RO+ cells, probably through activation of p56<sup>Lck</sup>, which also lessens CD4 endocytosis. These differences in p56<sup>Lck</sup> activation may be direct consequences of the CD45 isoform present, although the existing evidence suggests the hyper - reactivity of CD45RO+ cells is important in syncytium formation and is multifactorial in origin<sup>1, 2</sup>.

Enhanced calcium signalling or decreased downregulation of CD4 on exposure to gp120 may be germane to the enhanced syncytium formation seen in CD45RO+ cells. Increased expression of LFA - 1 on CD45RO+ cells does not appear to be a major factor in this, since changes in CD45 isoform expression alone on a T cell line are enough to affect syncytium formation. Increased affinity of integrins on CD45RO+ cells may be a direct consequence of CD45 isoform expression, although it remains possible that CD45 itself can interfere physically with the proximity of syncytium partners and affect the kinetics of this process. Data suggest that cell free HIV infection is more efficient in CD45- T cells, although this is complicated by the finding that CD45 may inhibit the transcription factor NFκB<sup>3</sup>.

Fresh CD45RO+ and, particularly, CD45RA+ CD4+ cells require activation, either *in vivo* or *in vitro*, to reverse transcribe and integrate HIV genome. Even when activation follows infection, there is defective uptake of HIV RNA in CD45RA+ cells, consistent with their impaired in fusion with infected cells.

Fresh CD45RA+ and CD45RO+ CD4+ cells express TNF receptors poorly and TNF does not induce ROIs or NFκB. Hence TNF is unlikely to break latency in completely quiescent cells. On activation, TNF receptor expression is up - regulated and TNF can modulate the effects of co - stimuli. TNF then co - stimulates proliferation in both sets of cells, but in CD45RO+ growth is inhibited, at high concentrations by a novel mechanism, independent of apoptosis.

## Models Of HIV Pathogenesis.

HIV infects several different types of cell and evolves, *in vivo*, into a number of phenotypically distinct forms. Hence a number of pathogenic mechanisms may operate together or occur during the natural history of HIV infection.

A prevalent model of AIDS pathogenesis is that CD45RO+ cells are infected by HIV and destroyed early in HIV infection<sup>4</sup>, leading to a loss of recall responses. However, few circulating CD45RO+ lymphocytes are infected with HIV<sup>5</sup> and although the loss of recall responses in early HIV infection correlates with absolute CD45RO+ CD4+ cell numbers, this relationship is incomplete<sup>6</sup>, suggesting more than one mechanism comes into play.

In section 1.5.5. a number of proposed explanations for the early loss and infection of CD45RO+ CD4+ cells in HIV infection were discussed. The data presented here show that gp120 induces a larger calcium influx in CD45RO+ cells. It has been proposed that gp120 induced signals prime for apoptosis, which is executed by signalling through the TcR/CD3<sup>7-10</sup>. In this, case it may be expected that gp120 induces apoptosis priming more easily in CD45RO+ cells and that their lower threshold for activation could deliver the TcR/CD3 "death sentence" more readily<sup>11</sup>. When tested *in vivo* in mice with transgenic human CD4, high levels of gp120 and high titre anti - gp120 were required to induce apoptosis<sup>10</sup>. Furthermore, it was subsequently found that apoptosis or activation associated lymphocyte death in human HIV infection does indeed predominantly affect cells expressing CD45RO, but largely in the CD8+ population<sup>12-14</sup>. These data suggest that gp120 mediated mechanisms do not contribute to apoptosis in HIV infection. Although other mechanisms for apoptosis in HIV infection have been proposed, such as abnormal peripheral antigen presenting cell<sup>15</sup> and thymic epithelial cell<sup>16</sup> function, but the likely explanation is that apoptosis is part of the normal immune response to viral infections<sup>14</sup>. Even if apoptosis is not an important mechanism for CD4+ cell depletion, gp120 may inhibit CD45RO+ cell signalling, contributing to the loss of recall responses.

A proposed mechanism for the greater replication of HIV in CD45RO+ cells *in vitro* is that they secrete more TNF and are more sensitive to TNF than their CD45RA+ counterparts - the so called "TNF autocrine loop"<sup>17</sup>. For this mechanism to be the major difference the infection of cells bearing different isoforms of CD45, viral entry, reverse transcription and integration should be the same in CD45RA+ and RO+ cells, which is not the case *in vivo*<sup>4</sup>. Additionally data presented in this thesis infer that TNF is not capable of inducing NFκB, the major transcription factor for HIV, in unstimulated CD45RA+ or RO+ CD4+ cells. The co - stimuli required to sensitise either population to TNF are capable of transactivating HIV in their own right. The concept that increased HIV replication induced by TNF can result in cell death in fresh T cells is unproven.

Another model of AIDS pathogenesis is based on minor homologies between gp120 and MHC Class II<sup>18</sup>, as would be expected from their binding to a common ligand. This model is based on the idea that the Class II homology drives an allogeneic response against cells expressing or binding gp120<sup>19</sup> resulting in CD4+ cell depletion

and a graft versus host like disease<sup>20</sup>. Like superantigens, this effect would be expected to produce a skewing of T cell receptor gene usage and although some HLA alleles are associated with a better prognosis<sup>21</sup>, no consistent pattern in TcR gene usage has emerged<sup>22-25</sup>. The graft versus host model is also incompatible with the poor prognosis seen in neonates infected *in utero*<sup>26</sup>. Superantigen activity of HIV has also been an irreproducible finding<sup>27, 28</sup> and probably does not contribute to AIDS pathogenesis.

These data show that fusion and reverse transcription are favoured in CD45RO+ cells and support and extend the data of Zack and Stevenson<sup>29-35</sup>, showing reverse transcription proceeding optimally in activated cells; CD45 isoform does not appear to be a limiting factor and hence CD45RA+ cells are capable of supporting these processes, so long as they are activated. It has been reported that exposure to HIV induces a switch from CD45RA to CD45RO expression, making originally CD45RA+ cells permissive to HIV infection<sup>36</sup>. Experiment presented here do not support the need for such a switch, because 1) resting CD45RA+ cells did not contain HIV DNA after exposure to the virus and 2), following exposure to HIV and then activation, cells contained HIV within a few hours, whilst up - regulation of CD45RO takes at least a day<sup>37</sup>.

The data in chapter 5 show that SF proceeds more effectively in CD45RO+ cells; this finding could be explained by the higher affinity of integrins on the CD45RO+ population, a consequence of the higher basal activation state or possible a direct result of the CD45RO isoform itself. The data on HPBALL cells suggest that CD45 isoform expression itself is a determinant of syncytium formation and viral fusion efficacy. DC and CD45RO+ cells associate readily in HIV infection, producing clusters and productive infection *in vitro*<sup>38</sup>, and this may be an explanation for the vulnerability of this population.

Hence fairly subtle differences between CD45RA+ and CD45RO+ CD4+ cells appear to enhance HIV infection of the latter. In one model of AIDS pathogenesis, HIV preferentially infects activated cells, expressing CD45RO<sup>4</sup>, MHC Class II<sup>30</sup> or IL2R $\alpha$ <sup>30, 39</sup>. Changes in *env* in HIV strains confer the ability to form syncytia in T cell lines or mitogen stimulated T cells<sup>40</sup> and are associated with a poor prognosis<sup>41-44</sup>. However, these strains tend to emerge only after a degree of CD4+ cytopenia has developed<sup>42,43,45,46</sup>, suggesting that other factors permit the outgrowth of these strains<sup>47</sup>. When a variety of HIV strains are tested for their ability to cause depletion of CD4+ lymphocytes in mice with severe combined immunodeficiency, SI strains were less effective than macrophage tropic strains<sup>43</sup>. Additionally, the rate of decline of CD4 lymphocyte numbers is linear from early in infection, with no enhancement late in infection<sup>48</sup>, suggesting the emergence of syncytium inducing strains does not hasten CD4+ lymphocyte destruction. This model of direct cytopathic effect of HIV has also been criticised because of the low level of infected cells *in vivo*, although lymph node material sometimes shows feature of syncytium formation<sup>49-52</sup>. Mathematical models, tentatively extrapolated from crude lymphocyte life span data, have been used to argue that regeneration of CD4+ lymphocytes may not keep up with even low level

cytopathic effect<sup>53</sup>. This in turn does not resolve how the rate of decline of CD4 numbers is lower at late stage disease, when viral titres are at their highest<sup>54</sup>.

Preferential infection of CD45RO+ cells and the expectation of cytopathic effect in this population fits the consensus view that there is an early decline in the number of CD4+ cells expressing CD45RO<sup>11, 55-58</sup>, MHC Class II<sup>59</sup> and IL2R $\alpha$ <sup>60-63</sup> and loss of recall responses<sup>64-66</sup>.

However, this model cannot account for the complete loss of response to neo - antigen seen in early HIV infection; responses to Hepatitis B<sup>67-71</sup> and Influenza<sup>72</sup> vaccine are impaired very early in HIV infection at a time when the CD45RA+ population is intact. This model also does not explain how CD45RA+ cells are eventually lost late in HIV infection<sup>11, 59, 73, 74</sup> or why *in vitro* responses to allogeneic or mitogen stimulation are also eventually lost<sup>65, 66</sup>.

Antigen presenting cell function is also abnormal in HIV infection, a result of HIV infection and depletion of monocytes<sup>59, 75</sup> and Langerhans/dendritic cells (DCs)<sup>76, 77</sup>, although the latter finding remains controversial<sup>78</sup>. Whilst monocytes retain the ability to present antigen until the onset of symptomatic infection, DCs become dysfunctional very early on<sup>79-81</sup>. Both populations of APC may become infected in the bone marrow at the CD34+ pluripotent stage<sup>82</sup>(unpublished data, S.Knight, M.Helbert), but DCs becoming rapidly defective because of their short life span. The defect appears not to be due to a simple reduction in numbers, since correcting APC numbers *in vitro* does not improve function. Although infected DCs have been shown to infect responding T cells<sup>38, 83</sup>, this observation has not been consistent<sup>84</sup>. Other abnormalities include defective cytokine secretion (see section 7.1 and <sup>85</sup>), prostaglandin secretion<sup>86-88</sup> and downregulation of MHC Class II<sup>89</sup>.

An alternative model has been developed, that takes into account the above findings<sup>11, 90-92</sup> and assumes that in adults the thymus does not compensate for peripheral loss of T cells and that T cell numbers are regulated by a balance between proliferation and activation associated lymphocyte death. A second, larger, assumption is that quiescent CD45RO+ cells re - express CD45RA, as discussed in section 1.4.2. Early loss of DC function prevents normal neo - antigen responses since naive T cells require DCs for presentation<sup>93-96</sup>, at least in the mouse. Responses to recall antigen may be lost more easily when antigen presentation is sub - optimal than those to allogeneic and mitogenic stimuli, explaining why recall is lost early. Since phenotypic switching to CD45RO is reliant on DC, the early decline in the CD45RO+ population may be a consequence of impaired recruitment to, rather than more death in, this compartment<sup>91</sup>. Although Rauscher leukaemia virus infects murine DCs and results in failure of T cell proliferation<sup>97</sup>, a human *in vivo* model is required to establish the role of DCs in AIDS pathogenesis. In a murine retrovirus model, DC abnormalities led to a switch to T<sub>H</sub>2 cytokine responses<sup>98</sup>. In humans a DC abnormality has been proposed as causing a putative shift to T<sub>H</sub>2 cytokine patterns<sup>99</sup>, although the existence of such an imbalance has subsequently been refuted<sup>100-102</sup>.

During early HIV infection a restricted number of cells are infected and viral isolates initially remain homogeneous. Subsequently a wider variety of cells become infected, dysfunctional and depleted and new quasi - species or strains of HIV emerge with greater virulence, for example escaping CTL surveillance<sup>103</sup> or anti - gp120 V3 loop antibodies<sup>104</sup>, conferring resistance to zidovudine<sup>41, 105-107</sup>, inducing syncytia<sup>46</sup> and producing *nef*<sup>108</sup>. It is thus likely that a number of pathogenic mechanisms operate together, particularly late after infection, in the pathogenesis of AIDS.

## Implications For Treatment.

Therapeutic agents may vary in their efficacy at different stages of HIV infection. For example, zidovudine is known to be effective *in vitro* in T cells and *in vivo* in late HIV infection<sup>109-114</sup> but fails to prevent disease progression in asymptomatic HIV infection<sup>115, 116</sup>. Nucleoside analogues have diminished *in vitro* efficacy against HIV in monocytes<sup>117</sup>, possibly because they contain less cytosolic thymidine kinase than lymphocytes<sup>118</sup>. Although anecdotal data suggest zidovudine may reverse HIV induced DC dysfunction<sup>119</sup>, the possibility remains that zidovudine is ineffective in early HIV infection because it does not prevent infection of antigen presenting cells<sup>120</sup>. This possibility is currently under exploration. The implication is that anti - HIV drugs need to be established in myeloid as well as lymphoid cells<sup>91</sup> to have a broad effects as possible on the full range of cells infected by HIV.

If APC dysfunction is important in early HIV infection, cytokines which potentiate APC function may be beneficial. For example  $\gamma$ IFN stimulates APC function in both monocytes and DCs. Although this cytokine has complex effects on HIV replication in monocytes<sup>121, 122</sup>, it decreases T cell HIV replication<sup>123</sup> and may have an additional effect in promoting T<sub>H</sub>2 responses. Trials of  $\gamma$ IFN in combination with zidovudine are underway.

The data presented in chapter 7 suggests that attempts to block TNF secretion and signalling may not be effective, since T cells must be already activated in order to receive TNF signals. Although there have been concerns that TNF mediates some of the systemic, non - infectious, features of HIV infection, this has been difficult to confirm, for example, TNF levels in individual patients do not correlate with the presence of cachexia<sup>124,125</sup>. The inhibition of proliferation noted in CD45RO+ cells occurred at high concentrations of TNF, which may not be obtained *in vivo*. TNF may even have beneficial effects in the killing of infected cells<sup>123, 126, 127</sup>. Hence attempts at blocking TNF signalling in HIV infection may be misguided and TNF may have limited beneficial effects.

The data presented in this thesis suggest many of the deleterious effects of HIV require T cell activation. Hence gp120 induced calcium signals are more pronounced in hyper - reactive CD45RO+ T cells and processes required for HIV infection of T cells - fusion / syncytium formation and reverse transcription - are carried out more effectively in activated T cells. Increase in HIV replication has been noted during coincident

infection<sup>128</sup> and vaccination<sup>129</sup> with agents known not to cause HIV transactivation. T cell activation can be reduced by avoiding infections and hence "safer sex" and "safer drug use" remain important strategies for delaying disease progression.

The use of immunosuppression remains a theoretical approach in HIV infection. Although anecdotal reports suggest the use of steroids in HIV infection can hasten the onset of symptomatic disease, a meta - analysis of the use of steroids in the treatment of HIV associated thrombocytopenia failed to show an acceleration of disease progression<sup>130</sup>. *In vitro*, steroids inhibit HIV transactivation on host cell stimulation<sup>130</sup>. The use of high dose, long - term steroids is likely to result in opportunist infections as well as suppressing residual anti - HIV activity. Cyclosporin has modest effects on inhibiting NFκB binding<sup>131</sup>, operating largely by inhibiting the induction of NFAT. Although cyclosporin does not inhibit HIV reporter gene transactivation<sup>132</sup> *in vitro*, it does have some beneficial effects on CD4+ lymphocyte numbers and delayed disease progression when used *in vivo* in HIV infection<sup>133</sup>.

*In vitro*, activated T cells can be eliminated using a anti - CD25 ricin combination<sup>39, 134</sup>; this has been used to clear infected T cells following *in vitro* infection. Continuous treatment for 3 days eliminates cells containing un - reverse transcribed virus, after which subsequent activation will not yield virus. *In vivo*, this technique would be limited by its inability to clear infected monocytes, but it illustrates how important activation is for HIV infection.

A slightly more specific means of targeting cells is to use transcription factor based specificity; NFκB is a transcription factor with activity restricted to lymphoid and some myeloid cells. Phosphothionate oligonucleotides are modified oligonucleotides with increased *in vivo* stability. When used to construct anti - sense oligonucleotide for the NFκB p50 and p65 gene initiation sites, they can ablate HTLV1 transformed tumours in mice<sup>135</sup> and hence may have affects against HIV. The use of these agents would presumably result in a degree of immunosuppression since NFκB is required for the induction of many human immunoregulatory genes. However they have potential when targeted at an HIV specific gene such as *Tat*. Attempts to link the HIV LTR to a gene causing single cell death in transgenic mice have yielded unexpected results<sup>136-138</sup>. Dendritic cells appeared to be most vulnerable to ganciclovir, suggesting that the HIV LTR is constitutively active in these cells and reiterating the precarious position of these cells in HIV infection. These genes are specific when linked to an HIV regulatory motif such as TAR and a retroviral vector could be used to insert such genes.

## Future Perspectives.

The role of CD45 in syncytium formation requires clarification, since it not only has implications for HIV pathogenesis but also for the intimate cell - cell interactions required for the normal immune response. Clones of cells stably transfected with isoforms of CD45 have been prepared which have comparable amounts of other surface

antigens (L. Goff, personal communication) and will be used to help determine whether CD45 simply acts as a molecular buffer or whether it modulates adhesion molecules.

Models of HIV pathogenesis make assumptions, based on incomplete *in vitro* or animal data, on the population dynamics of immunological memory. For example it is not certain, in adult humans, how much of the peripheral T cell pool size is maintained by peripheral proliferation and how much by continuing emigrants from the thymus, although existing data point to the former<sup>139</sup>. Existing means of determining lymphocyte life span<sup>140</sup> in man are limited by the need for radiotherapy (hence it not possible to study a healthy population) and the low numbers of cells carrying markers. What is required is a means of tracking reactive clones *in vivo*. Since primary responses can be detected *in vitro*<sup>141,142</sup> (Joyce Young, personal communication) and the technology for performing cell separation and limiting dilutions is well established, one possibility is to study cells *ex vivo* before and after vaccination. Serial monitoring of volunteers can determine the frequency of responding cells and their phenotypic characteristics. Such an approach may have implications for vaccinology, autoimmunity and for AIDS pathogenesis.

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