THE EXAMINATION OF MINIMAL REQUIREMENTS FOR PROLIFERATIVE RESPONSES TO ALLOANTIGEN BY NAIVE AND MEMORY T CELLS, DEFINED BY THE LEUKOCYTE COMMON ANTIGEN ISOFORMS.

submitted by

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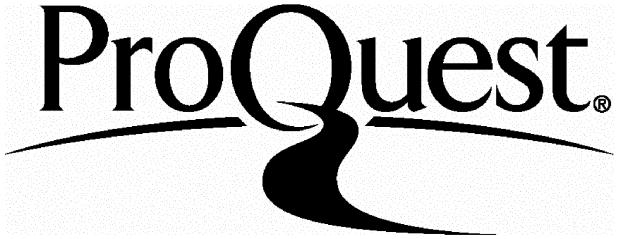

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ABSTRACT

Murine transfected fibroblast cell lines (TFCL) expressing HLA-DR1 (DR1) alone or co-transfected with CD58 or CD54 were used as allostimulators to investigate the minimal triggering requirements for CD45R subset allogeneic proliferation. Whole PBMC and CD45R subsets gave weak alloresponses to $DR1^+$TFCL. The proportion of responders was greater for PBMC than either of the CD45 subsets. Stronger alloresponses by PBMC and CD45R subsets against $DR1^+$CD54 and $DR1^+$CD58 TFCL were observed. Generally, $CD45RA^+$ and $CD45RO^+$ T cell subsets alone gave weaker alloresponses than whole PBMC. Recombining purified $CD4^+$ CD45R subsets after separation, led to responses similar in magnitude to whole PBMC, implying that the reconstitution of CD45R subsets has a co-operative effect mediated through either physical contact or cytokines. Alloresponses by PBMC and $CD4^+$CD45R populations to TFCL and LCL were all increased by the addition of IL-2. $CD45RA^+$ T cell responses to TFCL and LCL were significantly enhanced by the presence of IL-4, whereas in comparison, $CD45RO^+$T cells and not $CD45RA^+$ alloresponses were augmented by the addition of IFNγ. Whole PBMCs primed with $DR1^+$TFCL that normally produce low levels of proliferation induced vigorous responses when rechallenged with either $DR1^+$LCL or $DR1^+$TFCL. This is a strong indication that HLA-DR expressed on the TFCL is recognised by the responder T cell despite reduced primary alloresponses. The addition of autologous monocytes or CD28 MAbs to alloresponses involving TFCL or LCL increased proliferative responses by both $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ T cells.

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Facts are stubborn things.

-Alain Rene Lesage.

TO GRANDAD

ABBREVIATIONS

Ag	Antigen
AlloAg	Alloantigen
AMLR	Autologous MLR
ANOVA	Analysis of variance
APC	Antigen presenting cell
BrdU	Bromine deoxyuridine
Ca^{2+}	Calcium ions
ConA	Concanavalin A
cDNA	Cytoplasmic deoxy-ribose nucleic acid
CTLA-4	Cytotoxic T lymphocyte antigen-4
COH	Carbohydrate
CyA	Cyclosporin A
DAG	Diacylglycerol
DC	Dendritic cell
DN	Double negative
DP	Double positive
DR	HLA-DR
EDTA	Ethylene diaminetetra-acetic acid disodium salt
EGF	Epidermal growth factor
FCL	Fibroblast cell line
FCS	Foetal calf serum
FDC	Follicular dendritic cell
FITC	Fluorescein
GαM	Goat anti-mouse
GTP	Guanosine tri-phosphate
GvHD	Graft versus host disease
HBSS	Hanks balanced salt solution
HEV	High endothelial venules
HSV	Herpes simplex virus
ICAM-	Intracellular adhesion molecule
IFN-	Interferon-
Ig	Immunoglobulin

IL-	Interleukin-
IL-xR	Interleukin -x receptor
IP_3	Inositol triphosphate
KLH	Keyhole limpet haemocyanin
Ly	Lymphocyte
LAD	Leukocyte antigen deficiency
LCA	Leukocyte common antigen
LCL	Lymphoblastoid cell line
LFA-	Lymphocyte function antigen-
LN	Lymph node
Mph	Macrophage
MAb	Monoclonal antibody
MESF	Molecules of equivalent soluble flourochrome
MFI	Mean fluorescent intensity
Mg^{2+}	Magnesium ions
MHC	Major histocompatibility complex
MHC-Ag	MHC-antigen complex
MLR	Mixed lymphocyte reaction
MMC	Mitomycin C
MNC	Mononuclear cells
mRNA	Messenger ribose nucleic acid
MXH	Mycophenolic acid, xanthine and hypo-xanthine
NK	Natural killer cells
NMS	Normal mouse serum
O-/N-linked	oxygen-/nitrogen-linked
PALS	Periarteriolar lymphoid sheaths
PBS	Phosphate buffered solution
PBSA	Phosphate buffered solution with albumin & azide
PBMC	Peripheral blood mononuclear cells
PHA	Phytohaemagglutinin
PI-3K	1,4,5 Phospho-inositol kinase

PIP_2	Phosphoinositol 4,5 bisphosphate
PKC	Protein kinase C
PLCγ1	Phospho-lipase C gamma 1
PMA	Phorbel Myristate Acetate
PTK	Protein tyrosine kinase
PTPase	Protein tyrosine phosphatase
PWM	Pokeweed Mitogen
RA	CD45RA
RB	CD45RB
RC	CD45RC
RO	CD45RO
sAg	Superantigen
SI	Stimulation index
sol. Ag	Soluble antigen
Thy	Thymocyte
T_C	Cytotoxic T lymphocyte
TCR	T cell receptor
TFCL	Transfected fibroblast cell line
T_H	Helper T lymphocyte
VLA-	Very late antigen-

CHAPTER 1 - INTRODUCTION

T CELLS AND THE IMMUNE SYSTEM

Antigen specific T cell interactions are mediated by a membrane bound T cell receptor (TCR). All other receptor T cell interactions are antigen non-specific. The TCR is a heterodimer with each chain containing variable and constant regions. There are two types of TCR; αβ and γδ (Meuer et al. 1983; Hedrick et al. 1984). The majority of mature human T cells express the αβ form of the TCR, while less than 5% express the γδ TCR (Brenner et al. 1986). The TCR does not have sufficiently long intra-cytoplasmic domains for transmembrane signalling and therefore forms a complex with other membrane proteins known as the CD3 complex. The CD3 chains provide the TCR with the necessary transmembrane signalling apparatus (Weiss, 1993; Weiss and Stobo, 1984).

The TCR specifically binds to either class I or II Major Histocompatibility Complex (MHC), which is in a complex with a specific antigen peptide sequence. This complex is expressed on so-called antigen presenting cells (APC). A peptide of 8-20 amino acids long must be associated with MHC molecules in order to be recognised by the TCR (Christinck et al. 1991). The TCR will not bind to the peptide alone, the peptide groove in the MHC will contain nascent peptides if foreign antigen is absent (Gray, 1992; Christinck et al. 1991). T cells will bind to any cell expressing the appropriate MHC-Ag complex, however, co-expression of accessory or co-stimulatory molecules is necessary to induce maximum stimulation of the T cell and cause proliferation.

THYMIC T CELL DEVELOPMENT

All blood cells originate from a common progenitor cell called a stem cell. This cell will commit itself to a particular lineage depending on the micro-environment. Originating from the bone marrow, stem cells committed to T cell development migrate to the thymus for maturation (Koichi et al. 1992; Fowlkes and Pardoll, 1989; von Boehmer, 1990). The thymus is crucial in developing a T cell repertoire that is self-tolerant and MHC restricted before mature T cells migrate to the periphery. Developing T cells (thymocytes) can be divided into four main groups: (a) $CD4^-CD8^-$; (b) $CD4^+CD8^+$; (c) $CD4^+CD8^-$; (d) $CD4^-CD8^+$ (see Fig. 1-1). The most immature of these are the $CD4^-CD8^-$ (double negative) thymocytes.

These cells are the precursors to all mature T cells and are located in the thymic cortex (Bluestone et al. 1987; Kisielow and Miazek, 1995; Ceredig et al. 1983). Double negative thymocytes make up 5% of the total thymocyte population and have few surface markers. However, among the surface antigens they do express are CD1, CD2, CD5, IL-2R and CD7 (Crispe and Bevan, 1987; Lobach et al. 1985). They do not express TCR and cannot recognise or respond to antigen. Expression of such molecules is induced by interactions with the thymic stroma. The function of these molecules is unclear, although CD2 and IL-2R may be involved in the expansion of $CD4^-CD8^-$ thymocytes in the absence of TCR expression (Ceredig et al. 1983; Denning et al. 1988).

Extensive and rapid proliferation of $CD4^-CD8^-$ thymocytes is followed by a change in phenotype to $CD4^+CD8^+$ (Crispe et al. 1987; Petrie et al. 1990; Scollay et al. 1988). Double negative thymocytes begin to rearrange and express the β chain of the TCR, which is believed to be induced by IL-7R signalling. Rearrangement is closely followed by the expression of CD8 then CD4, double positive cells then rearrange the α genes and begin to express low levels of the αβ TCR. Rearrangement of the αβ TCR and the expression of the TCR/CD3 complex occurs on the cell membrane during the $CD4^+CD8^+$ stage, when cells are migrating from the cortex to the medulla (Scollay, 1991; Muegge et al. 1993; Peschon et al. 1994).

More than 95% of cortical thymocytes die before reaching the medulla through either of two selection processes (Rothenberg, 1992; Shortman et al. 1990; Scollay et al. 1988). Positive and negative selection are vital to T cell development, positive selection usually precedes negative selection although it is not a pre-requisite (Koichi et al. 1992; Fowlkes and Pardoll, 1989). The two selection processes occur in the thymus ensuring that the developing T cell repertoire is self-tolerant and MHC restricted (Fowlkes and Pardoll, 1989; von Boehmer, 1990).

Positive selection.

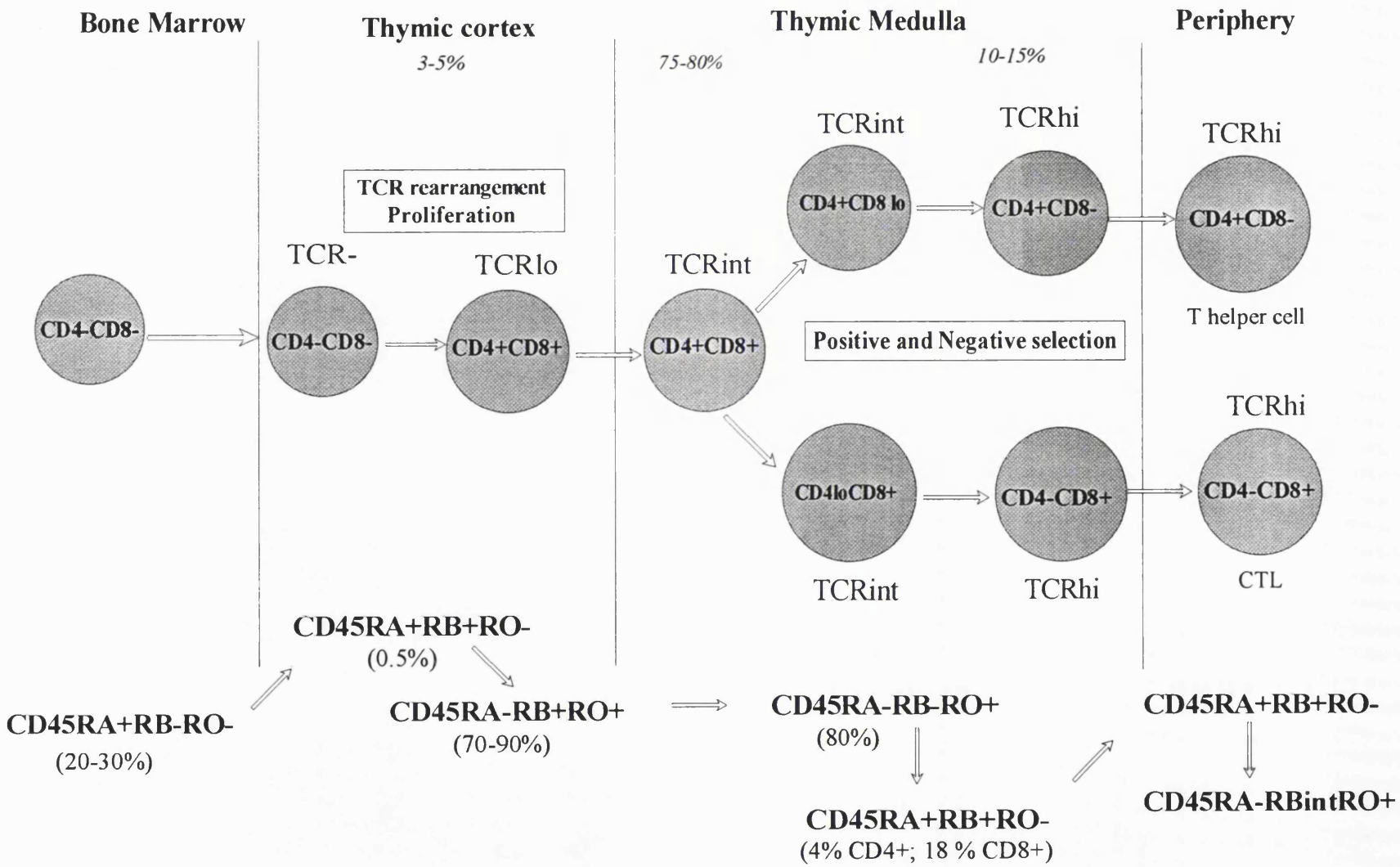
T cells must recognise antigen in the form of a peptide in a complex with self MHC class I or II. Positive selection occurs at the double positive stage of development when thymocytes begin to express low levels of TCR. Selection is thought to involve thymocyte interaction with thymic epithelium expressing self MHC in the apparent absence of foreign antigen.

Figure 1-1: Development of TCRαβ T cells.

Stem cells migrate from the bone marrow to the thymic cortex as $CD4^-CD8^-TCR^-$ $CD3^-$ thymocytes (Crispe et al. 1987; Crispe and Bevan, 1987; Petrie et al. 1990; Scollay et al. 1988). During thymic development, cells undergo gene rearrangement and express CD4 and CD8. Positive and negative selection occurs in the thymic medulla. Selected thymocytes increase their expression of TCR and lose either CD4 or CD8. Cells leave the thymus as single positive, mature T cells and enter the periphery. Numbers in italics represent the percentage of total thymocytes at each stage of development.

CD45 isoform expression by thymocytes varies according to the stage of development. The sequence shown is a proposed model based on the idea that CD45 isoform expression alters the signal transduction function of the TCR, e.g. expression of CD45RO increases the susceptibility of the thymocyte to activation (Fujii et al. 1992; Law et al. 1989; Gillitzer and Pilarski, 1990; Deans et al. 1991; Okumura et al. 1992; Fowlkes and Pardoll, 1989; Chang et al. 1991; Hathcock et al. 1992; Ezine et al. 1991). Numbers in brackets represent the percentage of thymocytes in each region of the thymus expressing the specified CD45 isoforms.

Fig. 1-1: Development of αβ T cells.



Interaction with self MHC rescues the thymocytes from apoptosis (Matzinger, 1993; von Boehmer and Hafen, 1993; Liu and Linsley, 1992). During these selection processes gene rearrangement of the α chain occurs. It is suggested that TCR preferentially associates with either CD4 or CD8 on the thymocyte and MHC molecules on the thymic epithelium. Cells in which TCR preferentially interact with CD4 molecules will eventually develop into mature CD4 T cells. Thymocytes whose TCR form stronger associations with CD8 become CD8 T cells. Cells destined to become CD4 cells will become exclusively restricted to MHC class II and CD8 to MHC class I (Chan et al. 1993a; Chan et al. 1993b; Davies, 1993).

Negative selection

To delete thymocytes expressing TCR responsive to the host, $CD4^+CD8^+$ thymocytes must undergo a second selection process. Self MHC reacting clones are deleted through an interaction with bone marrow derived cells expressing self MHC in the medulla. Negative selection will remove T cells with TCR of high affinity for self. Low affinity TCR will continue to the next stage of maturation (Robey and Fowlkes, 1994). Functional MHC, CD4 and CD8 are vital to thymic development (Ley et al. 1991). Without MHC, CD4 or CD8, thymocytes cannot undergo selection and therefore die. For instance, mice lacking CD4 or CD8 associated with the protein tyrosine kinase $p56^{lck}$ do not mature past the double positive stage (Molina et al. 1992; Grusby et al. 1991; Fung-Leung et al. 1991; Rahemtulla et al. 1991).

Induction of transmembrane signals through the TCR/CD3-CD4 (or CD8) complex must play a vital role during positive and negative selection and the development of fully mature T lymphocytes. Medullary thymocytes lose the expression of either CD4 or CD8 before proceeding into the final stage of development, where, they acquire helper or cytotoxic functions before emigrating to the periphery (Robey and Fowlkes, 1994).

T CELL SUBSETS.

The expression of different surface molecules identifies two major T cell subsets; T helper (T_H cells) and cytotoxic T (T_C cells). T_H cells express CD4, a surface protein known to associate with the TCR. CD4 binds to MHC class II molecules on APC (Lustgarten et al. 1991), facilitating adhesion between the two cells and signal transduction through the src kinase $p56^{lck}$ (Rudd et al. 1988; Glaichenhaus et al. 1991).

CD4 and CD8 weakly bind to MHC molecules stabilising the TCR-MHC interaction which in itself is relatively weak. MHC class II expressing cells process and present exogenous proteins that have been endocytosed by the APC. Monoclonal antibodies against CD4 inhibit T cell activation *in vivo* and *in vitro* indicating the importance of this molecule. $CD4^+$ T cells proliferate strongly in response to antigen and release cytokines that help B cell and macrophage activation (Kupfer and Singer, 1989).

The reciprocal population of T_C cells express CD8. This molecule is also physically associated with the TCR and binds to MHC class I molecules. Generally MHC class I^+ APC present endogenous proteins originating from within the APC, such as viral proteins. $CD8^+$cells also proliferate when activated but attach themselves to the target cell, deliver lytic granules into the cell membrane and detach; leaving the target cell to die. CD8 also possesses signal transduction apparatus in the form of $p59^{fyn}$. Both CD4 and CD8 are members of the Immunoglobulin (Ig) superfamily (Williams and Barclay, 1988).

T CELL ACTIVATION

Activation of T cells is an intricate process involving many ligand interactions between the T cell and APC or target cell, leading to two fundamental transmembrane signals. Binding of the TCR to the MHC-Ag complex is insufficient for T cell activation and induction of T cell proliferation. A second signal or costimulus is required as shown in Fig. 1-2 (Jenkins, 1992; Janeway and Bottomly, 1994; Bretscher, 1992).

The peptide presented by the MHC-antigen complex determines which antigen the T cell will respond to through the TCR/CD3 signal and is highly antigen specific. The accessory or co-stimulatory molecule signals are not associated with the TCR/CD3 complex and are antigen non-specific. The two signal model for T cell activation was first proposed by Bretscher and Cohn and is supported by a great deal of experimental evidence (Jenkins, 1992; Bretscher, 1992; Schwartz, 1990).

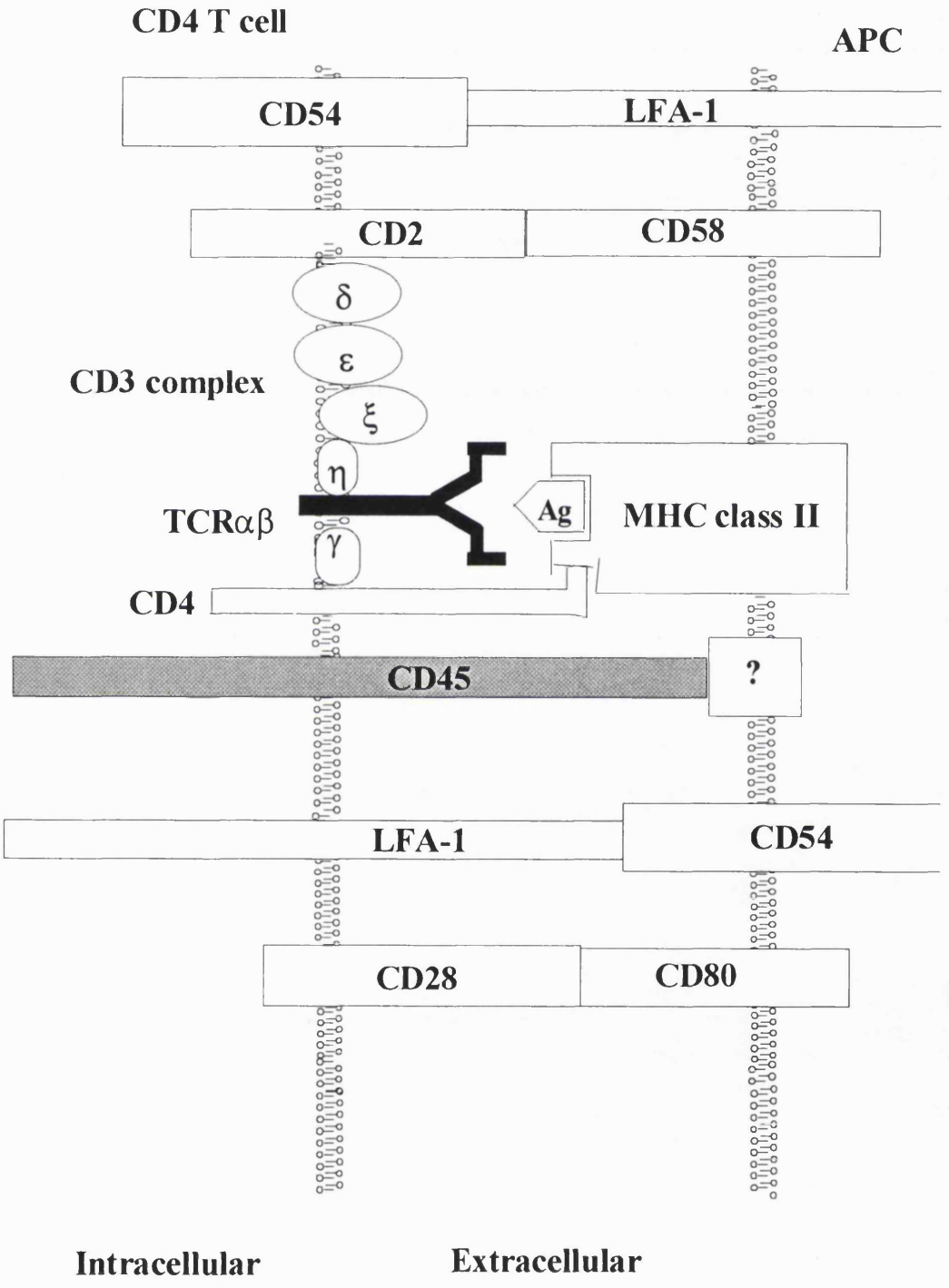
THE TCR/CD3 SIGNAL

The MHC-TCR interaction is antigen specific and triggers a cascade of intracellular signals (Fig. 1-3). The TCR has no intrinsic protein tyrosine kinase (PTK) and therefore interacts with cytoplasmic PTKs linked with other molecules such as CD4 associated $p56^{lck}$ or CD3 associated $p59^{fyn}$ (Gassmann et al. 1992; Cooke et al. 1991; Izquierdo et al. 1992).

Figure 1-2: Ligand interactions involved in T cell activation by an APC.

Communication between an APC and a $CD4^+$ or $CD8^+$ T cell involves a variety of ligand interactions in addition to the antigen specific TCR/CD3-MHC/peptide bond. Non antigen-specific ligand pairs form strong adhesive connections between the T cell and the APC in addition to activating co-stimulatory signals.

Fig.1-2: Ligand interactions involved in T cell activation by an APC



Although the final phenotypes and functions of $CD4^+$ and $CD8^+$ T lymphocytes are quite distinct, the signalling pathways employed are very similar, both leading to the activation of nuclear proteins.

CO-STIMULATORY SIGNALS

Delivery of a signal through the TCR/CD3 complex alone to a resting T cell leads to tolerance or unresponsiveness (Schwartz, 1992). A second 'co-stimulatory' signal is essential for full activation, proliferation and differentiation into an effector cell. A number of ligand interactions can induce a second signal.

The result of the two signals is the production of the cytokine IL-2 and expression of the IL-2 receptor which occur in the first 2-24 hrs of activation. This cytokine acts on the T cell in an autocrine fashion, promoting cell division and effector functions (Taniguchi and Minami, 1993).

CD2 and CD58.

CD2 is expressed on all thymocytes, T cells and NK cells and is another member of the Ig superfamily. CD2 is a 45-50 kDa transmembrane molecule that consists of external, transmembrane and cytoplasmic domains (Driscoll et al. 1991; Sayre et al. 1987; Yagita et al. 1988). The extracellular segment consists of two immunoglobulin- like domains (Dustin and Springer, 1989). The entire molecule is highly conserved between species particularly in the cytoplasmic region of rats, mice and humans (Yagita et al. 1988; Brown et al. 1988; Sayre et al. 1987).

CD58 has been identified as the principal ligand for CD2 and is widely expressed on haematopoietic tissues (Hunig, 1985). The interaction between CD58 and CD2 serves two main purposes:

a.) to augment adhesion between the T cell and the APC, target cell or cell-cell co-operation (Hunig, 1985);

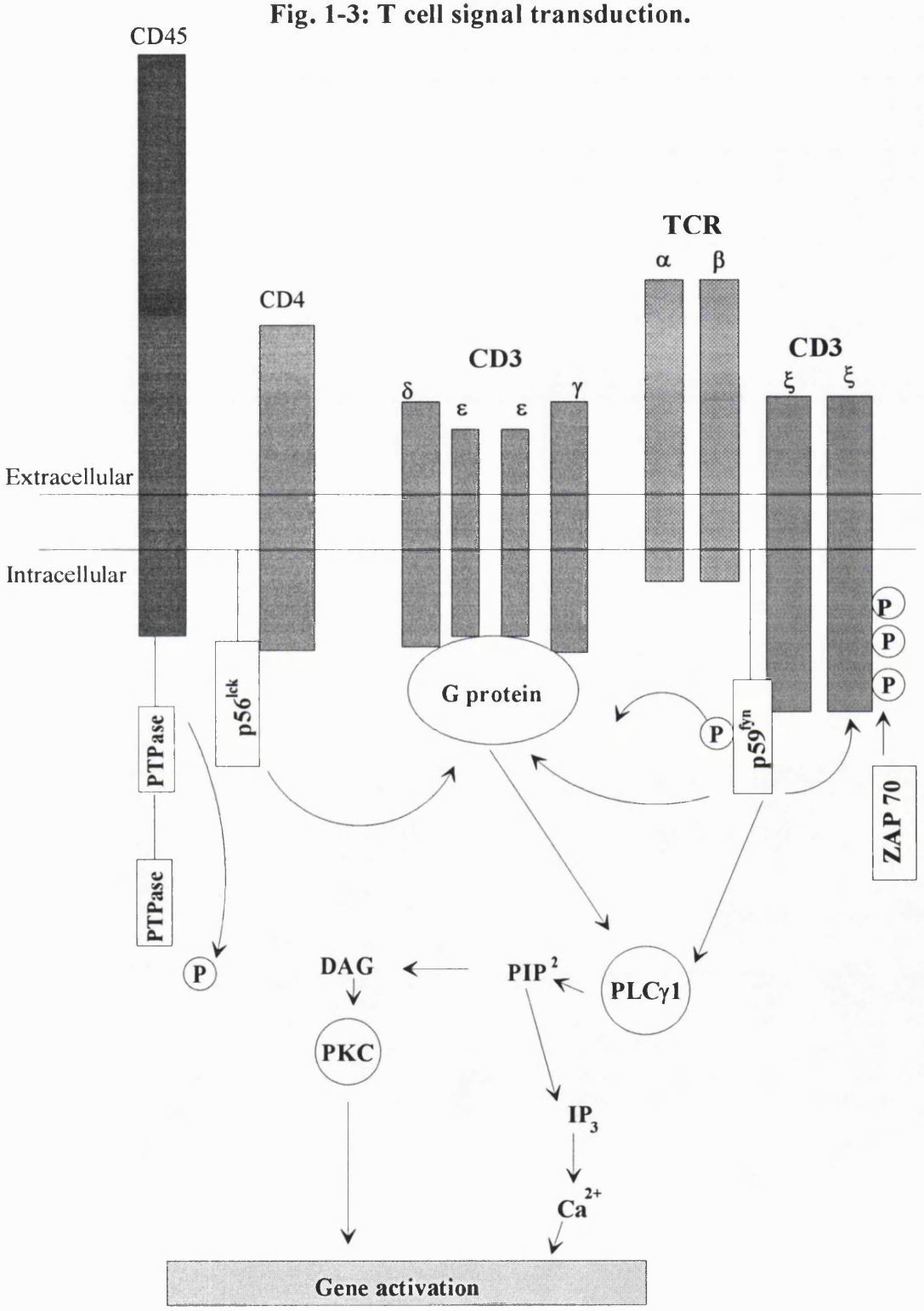
b.) the transduction of signals leading to T cell proliferation (Rothlein and Springer, 1986; Schraven et al. 1990; Beyers et al. 1989).

In rodents no homologue for CD58 has been identified and the principal ligand for CD2 is CD48 (Kato et al. 1992; van der Merwe et al. 1993). In humans, there is evidence to suggest CD48 and CD59 are also able to bind CD2 (Hynes, 1987; Hahn et al. 1993).

Figure 1-3: T cell signal transduction.

A schematic diagram of the multiple signalling pathways mediated by TCR/CD3, CD4 and CD45. Engagement of the TCR results in the formation of a complex with CD4 which binds to the MHC molecules. The protein tyrosine phosphatase (PTPase) CD45 dephosphorylates CD4 associated $p56^{lck}$ and $p59^{fyn}$ associated with the ζ chain of CD3 rendering these tyrosine kinases active (Trowbridge et al. 1992). Phosphorylation of the CD3 ζ chain by PTPase enables another PTK, ZAP 70 to bind to the phosphorylated molecule (Izquierdo et al. 1992). Two main pathways generate second messengers following T cell occupancy through PLC γ1 hydrolysis (Mustelin et al. 1990; Nel et al. 1995; Szamel and Resch, 1995). The first is through ZAP 70, the second is through a G protein or GTP binding protein transducing signal pathways from the TCR/CD3 complex to phospholipase C (PLC γ1), resulting in the hydrolysis of membrane bound phospho-inositol 4,5, bisphosphate (PIP_2). The products of PIP_2 hydrolysis, inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG) are the second messengers responsible for increases in intracellular calcium concentrations $[Ca^{2+}]i$ and protein kinase C (PKC) activation respectively (Berridge and Irvine, 1984; Imboden, 1988).

Fig. 1-3: T cell signal transduction.



Human T cells readily form rosettes with sheep red blood cells. E rosetting was the original means of identifying human T cells and is still used as a purification technique for T cells (Schwartz, 1992; Moingeon et al. 1989; Bierer et al. 1989). Human CD2 binds to the ovine CD58 in forming the rosettes because of the strong degree of homology with human CD58 (Rothlein et al. 1986). Chinese Hamster Ovary (CHO) cells transfected with CD58 produce T cell clusters that are blocked in the presence of monoclonal antibodies against CD2 or CD58. These studies support the notion that the CD2-CD58 interaction is an important adhesive contact between T cells and APC (Deckert et al. 1991). Although some studies have identified CD59 as a second ligand for CD2 (Hahn et al. 1992; Deckert et al. 1992), other groups have been unable to confirm this interaction (Arulanandam et al. 1993; van der Merwe et al. 1994).

CD2 is also believed to possess a signalling pathway that is capable of producing T cell activation, cytokine production and effector functions (Alcover et al. 1988; Linsley and Ledbetter, 1992; Beyers et al. 1989). Several transmembrane molecules including the TCR/CD3 complex, CD4, CD8 and CD45 have been shown to associate with CD2 on the surface of the T cell (Beyers et al. 1992; Schraven et al. 1990). Stimulation of T cells through CD2/CD58 binding leads to an increase in intracellular Ca^{2+}, protein kinase C (PKC) activation and production of second messengers (Kanner et al. 1992; Ley et al. 1991). There is also evidence showing that CD2 can directly activate src kinases such as *fyn* and *lck* (Carmo et al. 1993). The CD2-CD58 interaction is dynamic; stimulation through the TCR leads to rapid increases in CD2 avidity associated with the tyrosine kinase and PKC pathway. This has been described by Hahn as an example of 'inside-out' signalling (Hahn et al. 1993).

CD2 MAbs are grouped according to the epitope specificity. Antibodies directed against epitope I identify the CD58 binding site and prevent adhesion to APCs or E-rosette formation and do not induce T cell activation, although increases in intracellular Ca^{2+} does occur (Yagita et al. 1992). Antibodies against epitopes II and III are responsible for activation of T cells via an 'alternative pathway'. Epitope III is only present on activated T cells and is termed CD2R; it is a conformational determinant and does not require further protein synthesis to occur (Meuer et al. 1984; Linch et al. 1987).

Pairs of CD2 MAbs, each directed against a different epitope, can stimulate T cells leading to IL-2 production and effector functions. No single MAb against CD2 can induce T cell activation (Ledbetter et al. 1985).

In CD2 deficient mice, normal thymocyte development occurs, CTL responses and T_H function are normal. This may indicate that CD2 function is redundant and that other pathways replace the CD2-CD58 signal in the absence of CD2 (Killeen and Littman, 1993).

CD28 and CD80 (B7/BB1 family)

CD28 is believed to be a major co-stimulatory signal for T cells and is expressed by 80% of human T lymphocytes. It is a cell surface glycoprotein composed of 2 identical 44 kDa subunits and is expressed on virtually all $CD4^+$ cells. Expression of CD28 is variable on $CD8^+$ T cells (Schwartz, 1992; June et al. 1990; Lesslauer et al. 1986).

Cross-linking of CD28 in the presence of a mitogen or CD3 MAbs leads to T cell proliferation, CD28 alone has no effect on T cell activation (Ledbetter et al. 1985; Weiss et al. 1986; Lesslauer et al. 1986; Vandenberghe et al. 1992). Cross-linking is essential for activation through the CD28 pathway since studies using the CD28 Fab fragment exhibited an inhibitory effect (Harding et al. 1992).

CD28 signalling is believed to involve PI-3 kinase, PLC γ-1 and PKC activity which is similar to signalling via the TCR, although CD28 utilises different second messengers (Nunes et al. 1993). Based on the evidence that CD28 dependent IL-2 production is cyclosporin (CyA) and FK506 resistant, it follows that this pathway does not involve calcineurin, cyclophilin or the FK506 binding protein (Liu and Linsley, 1992; Vandenberghe et al. 1992). Other cytokines such as IL-1β IL-4, IL-5, IL-6 and IL-13 production are also affected by cross-linking of CD28. The delayed degradation of cytokine mRNA, causing prolonged cytokine release has been suggested as a possible mechanism for CD28 signal transduction (Ward et al. 1993).

T cell adhesion to CHO cells transfected with CD28 was one method employed to identify ligands for CD28 (Linsley and Ledbetter, 1993). MAbs against a surface glycoprotein CD80 (B7/BB1) were found to prevent formation of T cell clusters with the CHO cells and block mitogen induced T cell activation.

CD80 is constitutively expressed on spleen cells and mature DCs but can be induced on T cells, B cells, DCs and monocytes through activation. CD80 is a 50 kDa heavily glycosylated membrane protein and a member of the Ig superfamily (Freeman et al. 1991).

Knock-out mice lacking functional CD28 were found to have normal T cell development but severe defects in cytokine production. The number of $CD4^+$ and $CD8^+$ T cells were normal, as was CTL generation. Although not severely immunodeficient, these mice showed that CD28 plays an important role in T-B cell interactions (Shahinian et al. 1993).

When the same studies were carried out in CD80 knockout mice few defects in the immune system were found, suggesting that CD80 was not the only ligand for CD28. Subsequently another ligand has been identified, B7-2 or CD86 (Freeman et al. 1993). All members of the B7 family described in the literature so far are members of the Ig superfamily and express IgV like domains. Both CD80 and CD86 are induced on B cells with differing peak expression times after activation; CD86 is induced at 48 hrs following activation and CD80 can be days later suggesting that CD86 is the major ligand for CD28 during the initial activation (Boussiotis et al. 1993). It is still unclear whether these 2 ligands bind to the same site on CD28 or whether the signals they transduce are similar (Molina et al. 1992). Recent studies suggest that CD80 and CD86 differ in their ability to induce T cell differentiation into T_H1 or T_H2 type cells (Thompson, 1995). A second ligand for CD80 and CD86 is CTLA-4 (Harper et al. 1991), a molecule similar in structure to CD28 (31% homology at the amino acid level), but CTLA-4 expression occurs only on activated T cells and always at a lower density than CD28 (Ardavin and Shortman, 1992; Hathcock et al. 1993). The binding affinity of CTLA-4 is 20-fold greater than that of CD28 for the same ligand. CTLA-4 is found in equal amounts on $CD4^+$ and $CD8^+$ T cells and is associated more with amplification of responses than the initial priming (Bretscher, 1992).

To summarise, the CD28-CD80 interaction initiates vital signalling pathways that are independent of TCR/CD3 signals, resulting in CyA resistant IL-2 production and effector functions.

CD11a/CD18 and CD54

CD11a/CD18 or LFA-1 is a member of the integrin family, a group of membrane bound molecules whose main function is cell-cell adhesion (Dustin and Springer, 1989). All members of the integrin family are heterodimers consisting of an α subunit and a β subunit: CD11a (180 kDa) and CD18 (95 kDa) respectively in the case of LFA-1. The subunits are non-covalently associated in the presence of a divalent cation such as Mg^{2+} to stabilise the molecule (Hynes, 1987). Patients deficient in the β subunit (Leukocyte antigen deficiency patients) are subject to life threatening fungal and bacterial infections indicating the importance of these molecules in the immune system.

Almost all leukocytes express varying densities of LFA-1 although strong expression is limited to T cells, APC's and endothelial cells (Hynes, 1992; Sanchez-Madrid et al. 1983). LFA-1 has been described in mice and rats as well as humans. The murine LFA-1 shows a high degree of homology to human LFA-1 (Collins et al. 1994).

Three ligands have been identified for LFA-1; ICAM-1 (CD54), ICAM-2 (CD102) and ICAM-3 (CD50) (Binnerts et al. 1994). All 3 are members of the Ig superfamily and a high percentage of homology exists between them (CD54 and CD102 are 35% identical; CD54 and CD50 are 52%). CD54 and CD50 have five Ig-like domains whereas CD102 only contains two. Despite their structural homology, none of the molecules bind to the same epitope on LFA-1 (Martino et al. 1993; Marlin and Springer, 1987).

Tissue distribution of the ICAM's is also very different. CD54 is constitutively expressed by some tissues but is up-regulated on T cells by inflammatory cytokines such as IL-1 and IFNγ (Dustin et al. 1986). CD54 is involved in the recruitment of lymphocytes to areas of damage or inflammation and is believed to be the principle ligand for LFA-1 (Marlin and Springer, 1987; Kishimoto et al. 1989). CD102 is expressed on leukocytes and ECs. Unlike CD54, CD102 is not readily up-regulated by cytokines. The third ligand, CD50 is only found on leukocytes (resting and activated) and is thought to bind cells of lower LFA-1 density than $CD54^{+}$ cells (Marlin and Springer, 1987; de Fougerolles et al. 1991; de Fougerolles and Springer, 1992; Fawcett et al. 1992).

The interaction between LFA-1 and it's ligands is involved in T cell mediated killing, T_H and B cell responses, NK function and homotypic adhesion. All these interactions are dependent on the presence of Mg^{2+} (Springer, 1990; Kishimoto et al. 1989).

LFA-1 occurs in two forms, a resting and an activated state. Most resting T cells express the low activation state LFA-1, which facilitates loose associations with other cells such as ECs and APCs. If specific binding through TCR occurs, LFA-1 becomes activated, strengthening the bond between LFA-1 and its ligand by a conformational change (Dustin and Springer, 1989). The affinity of CD54 for LFA-1 remains constant. Conformational changes in LFA-1 are separate from the binding sites of CD54, CD102 and CD50 (Hogg, 1989; Linch et al. 1987).

The β chain of LFA-1 is susceptible to phosphorylation during activation. It is possible that this signal initiates the changes in LFA-1 and is described as 'inside-out' signalling. CD45 is thought to negatively regulate LFA-1 phosphorylation, although there is also evidence to suggest that in thymocytes, CD45 stimulates LFA-1 mediated adhesion (Arroyo et al. 1994). CD2 is also thought to influence the binding state of LFA-1 (Killeen et al. 1992), since LFA-1 can also be activated by G proteins and PTK (Hermanowski-Vosatka et al. 1992). This would be an example of 'outside-in' signalling.

LFA-1 is a substrate for PKC dependent phosphorylation. Interaction with CD54 leads to intracellular signals such as prolonged inositol phospholipid hydrolysis and increases in intracellular Ca^{2+}. It appears that engagement with ICAM molecules initially provides a cell-cell contact although there is evidence for a role as a co-stimulus (Pardi et al. 1992a; Littman, 1989; Pardi et al. 1992b; Haverstick and Gray, 1992).

THE LEUKOCYTE COMMON ANTIGEN-CD45

The leukocyte common antigen (LCA) or CD45 as it is more recently known, is a group of glycoproteins expressed on almost all haematopoietic cells. It was first characterised in the mid-1970's as a major transmembrane glycoprotein (Battifora and Trowbridge, 1983). In 1986, LCA was clustered as CD45 at the 3rd International Workshop and conference on Human Leukocyte Differentiation and further defined in 1989 (Schwinzer et al. 1992; Flanagan et al. 1986).

CD45 maps to chromosome 1 in both humans and mice. This gene can produce a potential of 8 mRNA's by alternative splicing of 3 exons - 4, 5 & 6 (Table 1-I); six of these have been isolated as cDNA and sequenced (Streuli et al. 1987). More recently, was used to identify products from alternative splicing of exons 7 and 8. These isoforms have not been detected as protein so far (Chang et al. 1991). The heterogeneity and antigenicity of CD45 is due to its isoforms and they vary in size from 180 kDa (RO) to 220 kDa (RA). All exons are located in the extracellular domain of the protein (Hall et al. 1988) and may be significant in modulating ligand interaction, as shown in Fig. 1-4A and Table 1-I (Trowbridge, 1991).

The majority of B cells and NK cells express the highest MW isoform of RA (Trowbridge, 1991; Thomas, 1989). In T cells, CD45 expression is very heterogeneous (see Section 1.8).

GENERAL STRUCTURE OF CD45

Most of the primary structure has been determined by cDNA analysis revealing a molecule with a MW ranging between 180 and 220 kDa depending upon which exon is expressed (Thomas, 1989). The extracellular region is a large, heavily glycosylated, amino terminal of between 400 and 500 amino acid residues and extends 28 nm from the cell membrane when no exons are expressed (Fig. 1-4B). This is extended a further 23 nm if RA, the largest exon is expressed (McCall et al. 1992; Ralph et al. 1987). CD45 is found in great abundance on the cell surface.

Table1-I:Correlation between exon expression and MAb detection.

Isoform	Exons expressed	Mabs staining positive for CD45R isotypes:			
		A	B	C	O
*ABC	3,4,5,6,7	+	+	+	
*AB	3,4,5,7	+	+		
AC	3,4,6,7	+		+	
*BC	3,5,6,7		+	+	
A	3,4,7	+			
*B	3,5,7		+		
*C	3,6,7			+	
*O	3,7				+

* Isoforms detected as cDNA in humans.

The size of the molecule and its highly dense expression on T cells means that it is likely to be one of the first molecular contacts between cells. Therefore changes in CD45 isoform expression may dictate which type of cells the lymphocyte comes into contact with and what extracellular signals are received (Alexander et al. 1992).

The extracellular portion contains many sites for O- and N-linked glycosylation, almost all of which are located in serine / threonine rich regions. Changes in exon expression are linked to changes in glycosylation of the extracellular portion (Thomas, 1989). This region also contains a conserved core motif, rich in cysteine residues that could act as a ligand binding site. To date, no ligands have been identified for any of the CD45 isoforms. Some studies suggested that CD22 was a possible ligand for RO (Stamenkovic et al. 1991) but this has now been widely refuted as CD22 binds to a moiety of RO that is common to many membrane molecules expressed by T cells; α 2-6 sialic acid (Engel et al. 1993). Attempts to identify potential ligands using a variety of methods have been unsuccessful.

The transmembrane region is a single chain of 22 residues. This region demonstrates no variability with isoform expression and is highly conserved between species. The cytoplasmic portion is a very large ($\approx$ 700 aa) and is also a highly conserved region of CD45. The size of the intracellular region would indicate an involvement in cell signalling. The primary and secondary sequence have close homology with the epidermal growth factor (EGF) receptor (Engel et al. 1993; Doolittle, 1981). Isolation of the human placenta protein tyrosine phosphatase (PTPase) in 1989 was shown to have 40% homology with the tandem repeats in the carboxy terminal of CD45 (Charbonneau et al. 1989). CD45 isolated from human spleen cells was found to contain intrinsic PTPase activity (Charbonneau et al. 1988; Tonks et al. 1990) which originated from the two internally homologous domains (tandem repeats), in the cytoplasmic domain, each consisting of $\approx$300 aa. This region is subject to phosphorylation on serine residues and is a substrate for protein kinase C (PKC) (Thomas et al. 1985; Koretzky et al. 1990; Ledbetter et al. 1988). Phosphorylation of either the first or second domain may alter enzyme activity and substrate specificity (Weaver et al. 1992; Koretzky et al. 1990).

Figure 1-4A: The exons of CD45.

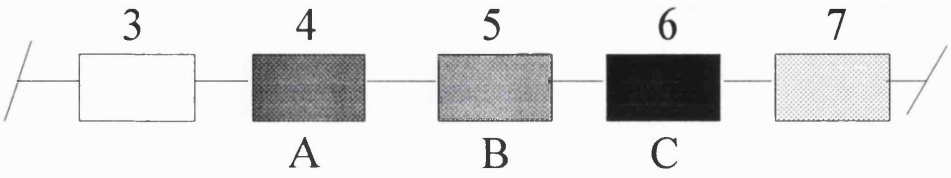
Expression of one or more of the exons of CD45 produces various isoforms of the CD45 glycoprotein. Splicing of exons 4-6 produces variations the extracellular domain of CD45. Expression of all exons produces the largest isoform of CD45 (CD45RA); expression of none of the exons produces the smallest isoform of CD45 (CD45RO).

Figure 1-4B: The structure of the CD45 glycoprotein.

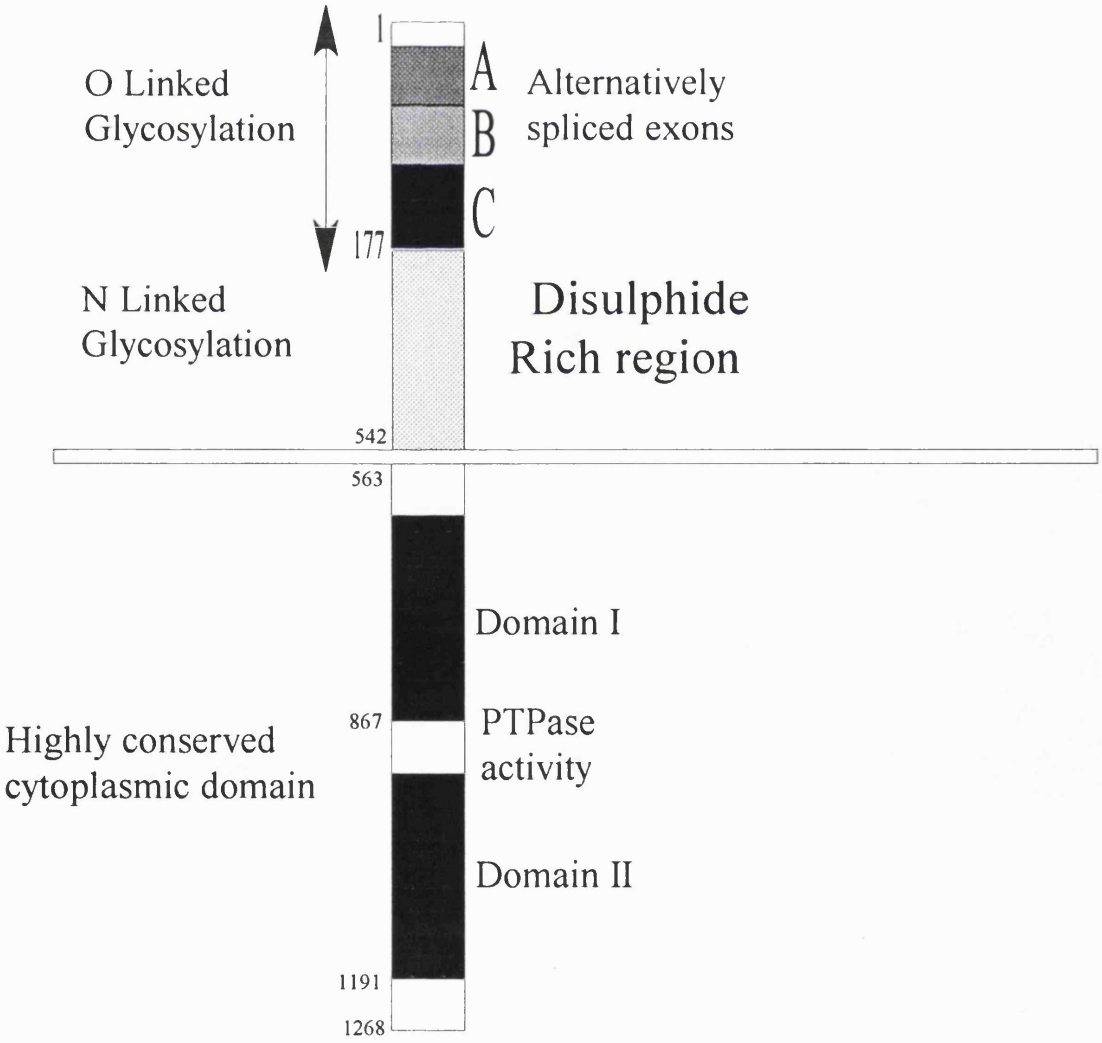
A, B and C represent the variable region of CD45 produced by the alternative splicing of exons in the CD45 gene. Domains I and II contain protein tyrosine phosphatase (PTPase) activity, responsible for phosphorylation of CD4 or CD8 associated $p56^{lck}$ and CD3 associated $p59^{fyn}$.

Fig. 1-4:Gene & protein structure of CD45

A. Exons of CD45



B. Protein domains of CD45



CD45 AND T CELL SIGNAL TRANSDUCTION.

CD45 appears to be essential for antigen induced T cell proliferation. Mutant cells that do not express CD45 have impaired proliferative responses that can be up to 100-fold lower than $CD45^+$ T cells. Activity is restored if CD45 is transfected into the mutant T cell line or if a $CD45^-$ reverts to a $CD45^+$ clone (Pingel and Thomas, 1989; Weaver et al. 1991). The PTPase activity of CD45 appears to be essential for signal transduction through the TCR. CD45 acts by dephosphorylating the tyrosine residues of its substrates (Justement et al. 1991).

An increase in protein tyrosine phosphorylation is one of the earliest detectable biochemical responses in T cell activation (June et al. 1990). CD45 is coupled to the TCR signals and associates with the phospho-inositol second messenger system, PIP_2 (Tonks et al. 1988; Tonks et al. 1990). Possible substrates for CD45 PTPase activity are $p56^{lck}$ that associates with CD4 and CD8 and $p59^{fyn}$, associated with the CD3 ζ chain. All of these pathways are uncoupled in the $CD45^-$ HPB-ALL T cell line suggesting the involvement of CD45 in early signal transduction (Deans et al. 1992). It is becoming increasingly clear that a variety of membrane receptors from the TCR to costimulatory molecules to cytokine receptors all use a variety of tyrosine kinases and PKCs to induce effective signalling. The co-association of molecules on the cell surface is paralleled by a co-association of appropriate enzymatically active molecules within the cell.

Knock-out mice, deficient in exon 6 of CD45, have less than 20% of normal circulating lymphocytes. These mice are profoundly immunosuppressed, with impaired thymic development, increased numbers of double negative thymocytes and low levels of double positive thymocytes. T_H function and proliferative responses against CD3 MAbs or mitogens are also impaired. No CTL responses can be detected. Clearly, CD45 is a vital component for normal T cell function and thymic development (Kishihara et al. 1993).

CD45 ISOFORM EXPRESSION

The discovery of MAbs against CD45 isoforms in humans, mice and rats has enabled many functional and phenotypic studies on T cell populations to be carried out. In humans, mature $CD4^+$ and $CD8^+$ T cells can be sub-divided on the basis of CD45R expression (Byrne et al. 1988; Sanders et al. 1988).

Although RA (220 kDa) and RO (180 kDa) isoforms are the most commonly used to define reciprocal populations, T cells are not exclusive in their expression of isoforms. A single T cell may express varying amounts of two or three isoforms at any one time. Approximately 10-30% of circulating T cells will express low intensities of RA and RO on their surface (Wallace and Beverley, 1990). The overall density of CD45 does not fluctuate a great deal, although the type of isoform may vary. RA^+ T cells also express high levels of CD45RB and -RC. The intensity of CD45RB and -RC is decreased on RO^+ cells (Zapata et al. 1994; Salmon et al. 1994).

Phenotypic differences between RA^+ and RO^+ cells are not limited to CD45 isoform expression (Sanders et al. 1988). RO^+ cells express higher amounts of adhesion and activation molecules that are only detected at low levels on RA^+ cells (Table 1-II) . Differences in adhesion molecules are indicative of the differences in circulation patterns and the cell types that the subsets communicate with (Wallace and Beverley, 1990). The levels of such markers may also be a reflection of the activation state of the cell (Smith et al. 1986). Initially, RA^+ and RO^+ populations were thought to originate from distinct lineage's based on their ability to help B cells. RA^+ cells were termed the 'helper-inducer' subset while RO^+ cells were the 'suppressor-inducer' cells (Sanders et al. 1988). Further functional studies such as differing responses to recall antigens, suggested that these populations were more likely to be different maturation stages of the same lineage (Beverley, 1990).

In mice, CD45RB divides T cells into $CD45RB^{hi}$ and $CD45RB^{lo}$ subsets with similar properties to human RA and RO populations respectively (Sanders et al. 1988). Rat T cell populations are identified by their expression of CD45RC (Sarawar et al. 1993). To date, no MAbs against murine RO have been found, failure to identify an murine CD45RO Mab raises the question of whether this isoform exists in the mouse or rat and alternative splicing of CD45 only produces the higher Mwt isoforms.

CD45 ISOFORM EXPRESSION AND FUNCTION.

When T cells are divided on the basis of RA and RO expression, many phenotypic and functional differences can be identified (Table 1-II). A number of these differences give support for a 'naive-memory' T cell hypothesis, initially suggested by Morimoto's group (Morimoto et al. 1985).

The critical functional difference between RA^+ and RO^+ cells is the ability to respond to recall antigen (Morimoto et al. 1985; Sanders et al. 1988). RA^+ cells do not proliferate to this type of antigen whereas RO^+ cells respond vigorously. This has led many to believe that RO^+ T cells represent memory T cells. Further evidence is provided by the increased expression of adhesion / co-stimulatory molecules on the surface of RO^+ cells (Beverley, 1990). These molecules may increase the efficiency and speed of signal transduction although no direct evidence for this exists.

Responses to CD2 and CD3 MAbs are also higher in RO^+ T cells (Luqman and Bottomly, 1992; Byrne et al. 1988), another piece of evidence implying that RO^+ cells respond with greater efficiency or have higher affinity than their RA^+ counterparts. It is interesting in this context that the responses of RA^+ and RO^+ T cells are equal in responses to alloantigens (Morimoto et al. 1985).

Despite phenotypic and functional differences between CD45R populations that support the naive / memory T cell model, there are also many pieces of evidence that do not comply (Akbar et al. 1988; Akbar et al. 1989; Morimoto et al. 1985; Smith et al. 1986; Sparshott et al. 1991; Bell and Sparshott, 1990; Sarawar et al. 1993). At its simplest level, RA^+ T cells switch to RO^+ during mitogenic stimulation not involving TCR engagement (Trowbridge et al. 1992) and therefore without implying any antigen specificity. In the thymus, RO^+ thymocytes proliferate rapidly during selection in the absence of antigen (Serra et al. 1988; Fisher et al. 1990). It could therefore be argued that CD45 isoform expression is more likely to be an indicator of the activation state of a cell. Under these circumstances, expression of RA would indicate a resting cell and RO a recently activated cell.

More fundamentally, 'memory' T cells are less easily defined than 'memory' B cells. T cells, upon antigen driven stimulation, do not undergo further or post thymic TCR rearrangement but clonally expand and become activated. Whether this activated state is a uni-directional process and can be defined by CD45R isoform expression is a controversial issue.

Table 1-II: Phenotypic and functional differences between CD45R T cell subsets.

		$CD45RA^+$	CD45RO	Reference
Phenotype	CD45RB	High	Med/Low	(Akbar et al. 1994)
	CD45RC	High	Low	(Zapata et al. 1994)
	MHC class II	-	+	(Beverley, 1990)
	CD11a/CD18	+	++	(Kristensson et al. 1992)
	CD2	+	++	"
	CD54	-	+	(Beverley, 1990)
	CD58	+	+++	"
	CD25	-	+	"
	CD29	++	+++	(Sanders et al. 1988)
	CD7	++	+/-	(Heinrich et al. 1989)
	CD44	++	+++	(Buckle and Hogg, 1990)
	CD26	++	++	(Akbar et al. 1991)
	CD28	++	++	(Personal observations)
	L-selectin	+++	+	(Buckle and Hogg, 1990)
Function	Recall antigen	-	+++	(Beverley et al. 1992; Merkenschlager et al. 1988)
	Alloantigen	+++	+++	(Morimoto et al. 1985b)
	Mitogen	+++	+	"
	CD3	+	++	(Welge et al. 1993)
	Inhibition by CD25	++	+++	(Morimoto et al. 1985b)
	AMLR	+++	+/-	"
	CD2	-	+++	(Kanner and Ledbetter, 1992)
Cytokine Release	IL-2	+++	+	(Sanders et al. 1988; Budd et al. 1987)
	IL-3	+	++++	(Budd et al. 1987)
	IL-4	-	+++	(Ferrer et al. 1992; Luqman and Bottomly, 1992)
	IL-6	+++	+	(Beverley, 1990; Budd et al. 1987)
	IFNγ	+	++	(Sanders et al. 1988; Budd et al. 1987)
Lymphatic Circulation		Efferent	Afferent	(Mackay, 1991)

TRANSITION IN CD45 EXPRESSION

As previously discussed, thymocytes go through a number of stages before emerging into the periphery as mature T cells (Fig. 1-1). During this development, CD45 isoform expression also changes. In humans, the earliest thymocytes are $CD4^-$ $CD8^-$ (double negative) and do not express the CD3/TCR complex (Crispe and Bevan, 1987). These cells are RO^- with 20-30% expressing RA (Fujii et al. 1992). The most prominent population of thymocytes are $CD4^+$ $CD8^+$ (double positive) and express RO thus implying that thymocytes switch isoforms during their differentiation into double positive thymocytes and migration into the cortical region of the thymus (Fujii et al. 1992). RO expression remains during the differentiation into single positive $CD4^+$ or $CD8^+$ thymocytes (Peakman et al. 1992; Harris et al. 1992; Lightstone and Marvel, 1993; Rabian-Herzog et al. 1992). Cord blood and mature naive T cells are RA^+. Thymocytes must therefore revert back to RA before entering circulation as mature T cells. This switch is believed to be one of the final steps in T cell maturation (Fujii et al. 1992). In mice and rats the scenario is very similar and histological staining identifies the medullary thymocytes to be $CD45RC^-$ with reversion to $CD45RC^+$ within 7 days of entering the periphery. Again the most immature thymocytes in the thymic cortex express CD45RC (Law et al. 1989).

Pilarski *et al* suggest a model of thymic development whereby expression of RO is induced by negative selection. Continued expression of high MW isoforms of CD45 may be a pre-requisite for thymocytes to continue to mature T cells. Expression of RO may be important in the initiation of intra-thymic cell death (Pilarski et al. 1991; Okumura et al. 1992; Deans et al. 1989; Gillitzer and Pilarski, 1990; Liu and Linsley, 1992).

There is evidence to suggest that the loss of RA expression correlates with thymocytes undergoing positive or negative selection. This would indicate that thymocytes are activated during thymic selection hence the switch to RO. In vitro activation of RA^+ thymocytes induces transition to RO and cell death. If the proposed model is correct, selected thymocytes are rescued from cell death by reverting to RA expression. Exactly where this reversion occurs and how it is induced is unknown but may occur immediately before or after cells leave the thymus.

Evidence to support this view comes from CD45 isoform expression on cord blood T cells and the 'virgin' T cell population in the periphery. Both of which express RA and are thought to represent the most Ag inexperienced T cells. During activation of a resting 'virgin' T cell in humans, RA is down-regulated and RO is rapidly up-regulated. Greater than 90% of the initial population will express RO by the 7th day of activation and continue to do so for many weeks of culture. Peak expression occurs 140 hrs following the initial stimulus, indicating the need for mitosis to occur prior to the expression of RO. Contrary to RA^+ T cells, RO^+ T cells maintain expression of RO when activated and remain so for many weeks thereafter (Akbar et al. 1988; Smith et al. 1986; Byrne et al. 1988; Sanders et al. 1988; Clement et al. 1988; Rothstein et al. 1990; Serra et al. 1988; Lightstone et al. 1992). Initially, the conversion of RA→ RO was believed to be uni-directional. There is however, increasing evidence suggesting that mature T cells can re-express high MW isoforms of CD45 (Michie et al. 1992; Warren and Skipsey, 1991; Sarawar et al. 1993; Rothstein et al. 1991; Sparshott et al. 1991; Brod et al. 1989). In humans, a T cell line has been isolated that cyclically expresses RA and RO (Rothstein et al. 1991). In rats, the adoptive transfer of $CD45RC^+$ T cells to an athymic nude rat induces >20% of this population to switch to $CD45RC^-$ by day 21. If $CD45RC^-$ T cells are transferred, 70% revert back to $CD45RC^+$ by day 3. Here, a rapid and dynamic switch back to the 'naive' phenotype is seen that is accompanied by a change in function (Bell and Sparshott, 1990; Sparshott et al. 1991; Sarawar et al. 1993). It is argued that RO expression may identify an end stage cell and not a memory cell. Reversion to a high MW isoform may rescue a T cell from apoptosis (Akbar et al. 1993). Expression of RO on plasma cells may accord with this in B cell development (Justement et al. 1991).

PRIMARY T CELL RESPONSES.

Activation of a resting naive (RA^+) T cell by an APC expressing MHC-Ag complexes, results in rapid proliferation and expansion of the T cell. A primary response is thought to occur in the paracortex of the lymph node or periarteriolar lymphoid sheaths (PALS) of the spleen where naive T cells are particularly abundant (Springer, 1994; Picker and Butcher, 1992). An encounter with antigen by a naive cell results in expansion of the cells expressing TCR specific for the initial antigen-MHC complex.

These cells differentiate into effector cells and enter the circulation 3-4 days following the initial activation (Mackay and Imhof, 1993). Effector cells express the necessary adhesion molecules to enable movement in and out of blood vessels and tissues, in addition to a conversion from RA^+ to RO^+(Sanders et al. 1988). $CD4^+$ effector cells secrete cytokines that affect the growth and maturation of CTL and B cells. $CD8^+$ effector cells kill antigen specific target cells by lysis (Fig. 1-5).

Primary responses are usually short-lived and result in a rapid clearance of antigen from the body. However, some responses have been known to last for months. The length of time a primary response lasts, is dependent upon the persistence of the antigen in the body and the dose of antigen (Celada, 1971).

Following a primary response, the immune system is left with a surplus of redundant effector cells specific for a particular antigen. If these cells were to remain in circulation, progressive accumulation of effector cells would occur, leading to the dilution of naive T cells and a reduction in the T cell repertoire diversity. Once an immune response has subsided, effector cells follow one of three suggested pathways (Fig. 1-6); a switch back to RA^+ T cells, development of anergy or death by apoptosis.

Death by apoptosis.

A number of studies using superantigens and Vβ specific MAbs have shown that the majority of antigen specific cells disappear, leaving a very small number in circulation (Gray, 1992; Celada, 1971; Feldbush, 1973; Mackay, 1993). It is unclear how these cells are removed, but the favoured mechanism is by apoptosis in the spleen or gut (Sprent et al. 1993). Effector cells may have a pre-determined life span where programmed cell death occurs after a particular number of cycles. Immunofluorescent staining studies identified a reduction of the protein bcl-2 through progressive cell division (Akbar et al. 1993). The majority of RO^+ T cells are short lived compared to RA^+ T cells (Michie et al. 1992).

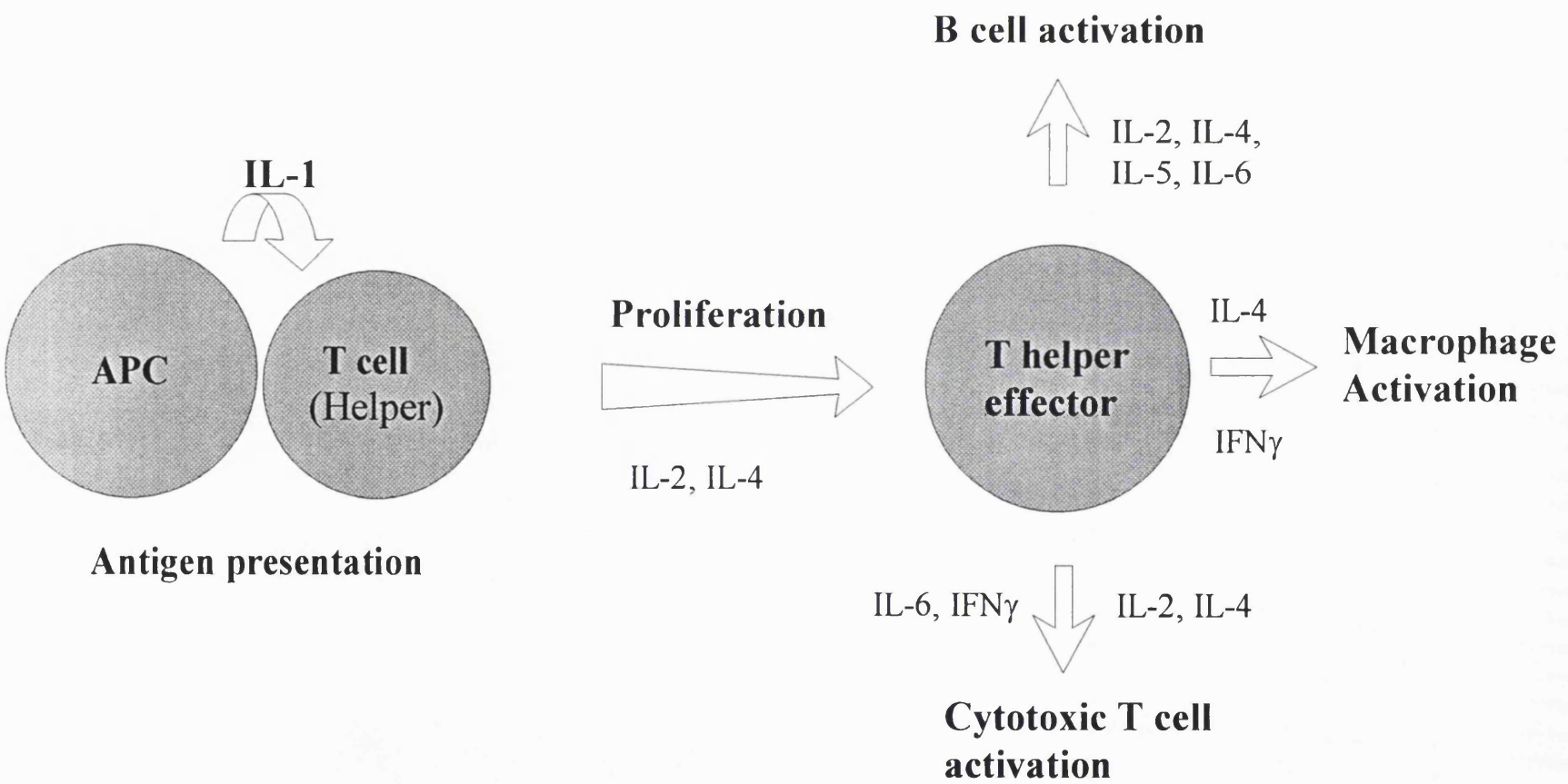
Generation of memory.

A number of hypotheses have been proposed to explain the generation of T cells (Fig. 1-6). It was historically assumed that T cell memory was carried by long lived recirculating lymphocytes. Evidence for this was found in mice. Memory T cells specific for *Mycobacterium tuberculi* were long lasting *in vivo* even following treatment with cyclophosphamide which prevents cell division (Orme, 1988).

Figure 1-5 : Helper T cell activation.

Presentation of antigens to $CD4^+$ T cells results in proliferation and the production of effector cells. The release of cytokines from effector $CD4^+$ T cells causes the activation of $CD8^+$ cytotoxic cells, macrophage activation and help for B cells. This could lead to excessive secondary responses and cause symptoms similar to toxic shock syndrome (Sprent 1994). Clearly these effector cells must be removed from circulation. Effector cells with short life spans are vital and would ensure that the immune system did not lose the ability to respond to new antigens throughout adult life. Elimination by apoptosis is thought to be one of the main physiological methods used to cull cell numbers in the immune system (Sprent et al 1993).

Fig.1-5: Helper T cell activation



T cell clones maintained in culture with IL-2 have been shown to differentiate into smaller resting cvells which can survive for many months in vitro without dividing.

Murine $CD8^+$ T cells can protect the host from viral antigen in the absence of persisting antigen, this protection can last for many years and is transferable (Gray and Matzinger, 1991; Mullbacher, 1994).

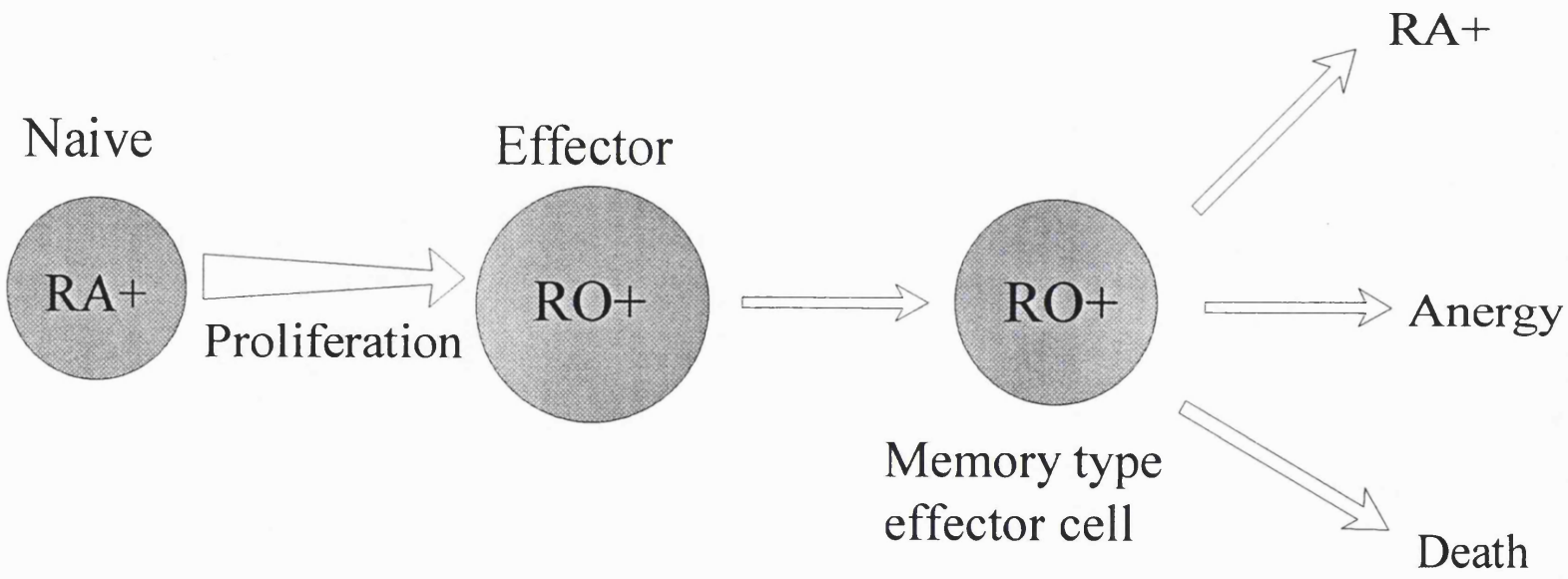
An alternative model for memory T cells, is the continual stimulation of short-lived clones. Gray and Matzinger demonstrated that primed $CD4^+$ and $CD8^+$ T cells were short lived in the absence of antigen using an adoptive transfer model (Gray & Matzinger, 1991). Analysis of T cell life spans also favours short-lived clones for RO^+ cells. The average life span of a T cell in rats is weeks, in humans it is between 18-30 months (Tough and Sprent, 1994; Freitas and Rocha, 1993; Mackay et al. 1990). When CD45R expression is taken into account, it was surprising to discover that equivalents of RO^+ cells divide more rapidly than RA^+ T cells in sheep, mice and humans. Maintenance of T cell memory by short-lived clones would require continual stimulation of these cells. The dose of antigen during the initial infection and the frequency of exposure may play a role in the production and maintenance of memory (Akbar et al. 1993). If, as evidence shows, persistence of antigen plays an important role in T cell memory, where would the antigen be stored? Follicular dendritic cells (FDCs) in primary follicles and germinal centres can store antigen in the form of immune complexes for up to a year after the initial infection (Mandel et al. 1980). This may be one way of continual stimulation of T cells but the antigen stores would eventually run out. In addition, few T cells are found in the germinal centre. B cells would have to process the antigen and present it to the specific memory T cell clone (Gray et al. 1991). No direct supportive evidence for this hypothesis has been found.

The type of antigen can also be a factor in that some antigens, particularly viruses, persist in the host following immune responses (Sprent, 1994). Herpes simplex virus (HSV) for example will lie dormant for many years following the primary response. Proliferation against any antigen leads to the localised release of cytokines by effector cells. Cytokines are not antigen specific and could therefore act on any T cell in the vicinity that may not be directly involved in the removal of that particular antigen. Release of IL-2 for example, may lead to general low level proliferation of localised memory cells, although this model implies that memory is maintained by the chance encounter of a proliferating effector cell (Panina-Bordignon et al. 1989).

Figure 1-6. The fate of effector T cells.

Once a primary response has subsided, three possible pathways have been proposed for the fate of effector cells. These pathways maintain homeostasis and prevent the immune system being overwhelmed by cells of a given specificity. The factors that determine which pathway is selected are unknown.

Fig.1-6: The fate of effector T cells.



Cross-reactivity is another suggested method of memory T cell maintenance. Memory T cells are more susceptible to activation than naive T cells. Some individuals have demonstrated a natural immunity to malaria through cross reactivity with T cells specific to tetanus (Currier et al. 1992; Fish et al. 1989). It is quite possible that long-lived clones and the continual stimulation of short-lived clones maintain T cell memory together. The type of antigen involved may determine which of the suggested models plays the most important role and how long memory lasts.

THE PHENOTYPE OF MEMORY T CELLS.

In vitro secondary responses are more vigorous than primary proliferation, this cannot simply be explained by the expansion of cells following a primary response. Secondary and even tertiary responses are thought to involve cells with greater specificity for the antigen than induced the initial primary response. Individuals displaying 'immunity' to a particular antigen following exposure indicates the possible existence of memory in the immune system. These facts support the existence of memory T cells. For a number of years, researchers have attempted to identify a cell population that may be responsible for immunological memory. The fact that RO^+ T cells respond to soluble recall antigens made these cells a strong candidate for the memory T cell (Beverley 1987). Subsequent studies have provided evidence for and against this argument. The key points for and against in this argument are discussed below.

Evidence that RO^+ T cells are memory cells.

RA^+ and RO^+ T cells differ in a number of ways, some of which identify RO^+ T cells as a population that are more readily activated than RA^+ cells (see Table 1-II). For example, the increased intensities of the adhesion/co-stimulatory molecules CD2, CD54 and CD58 would aid cell-cell contact and the strength of the transmembrane signals. RO^+ cells express TCR/CD3 complexes in physical association with co-receptors CD45 and CD4 or CD8 (Janeway and Golstein, 1992; Dianzani et al. 1992). These surface molecules are independent of each other on RA^+ cells. The pre-formed complexes would ensure increased efficiency and speed in signal transduction although no direct evidence for this exists. Basal levels of the second messengers such as DAG are also raised in RO^+ cells, another indication of RO^+ cell's readiness for activation (Robinson et al. 1993).

In vitro analysis of RA^+ and RO^+ response to various stimuli, demonstrate that RO^+ T cells are more sensitive to activation than RA^+ cells. In addition to the recall antigen responses, RO^+ cells proliferate more readily to CD3 and CD2 MAbs (Wallace and Beverley, 1990). Differences in the type of APCs employed by the two cell types are also evident. RA^+ cells require professional APCs such as dendritic cells (DC) but RO^+ cells can respond to antigen presented by activated B cells and macrophages in addition to DCs (Ronchese and Hausmann, 1993).

Recent thymic emigrants and the majority of cord blood cells express high MW CD45 isoforms (Peakman et al. 1992). In the case of humans this is RA. Thymocytes are thought to switch from RO to RA shortly before leaving the thymus (Fujii et al. 1992). If RA^+ cells are the 'naive' population, then a total thymectomy should result in a reduction of RA^+ cells in the periphery with age. Studies in the mouse reveal a decrease in high MW CD45 isoform (CD45RB) cells 10 weeks after a thymectomy and a failure to respond to new antigens (Kappler et al. 1974).

Decreases in the absolute number of cells of all phenotypes occur with age (Cossarizza et al. 1991; Franceschi et al. 1995). Comparisons between a young age group (19-25) and 75-84 year olds revealed that RA^+ cell numbers were significantly lower while RO^+ cells significantly higher in the old age group. Further analysis demonstrated a significant drop in the absolute number and percentage of RA^+ cells up to the age of 40 with little change thereafter. This decrease correlated with an increase in the number of RO^+cells (Nijhuis et al. 1994). The release of mature RA^+, naive cells from the thymus and the life span of new naive cells decreases with age. In mice, the number of naive cells specific for a particular antigen (e.g. KLH) decreases with age. All the above evidence demonstrated a correlation between decreasing naive cell numbers and age, although high MW CD45 isoform cells do not disappear altogether, even in the very old. Centurions for example, show a consistent number of $CD4^+$ and $CD8^+$ naive cells (Franceschi et al. 1995). An individual will never encounter a large majority of the antigens the cell repertoire caters for, this would account for a proportion of naive cells remaining in the elderly but not all. Although thymic remnants are found in individuals in their 90s, the release of thymic emigrants into peripheral blood decreases after the age of about 25.

Other lymphoid tissues such as the intestine may continue to produce fully matured T cells in the absence of a fully functional thymus and may be responsible for the release of new naive T cells in later life (Michie et al. 1992; Tough and Sprent, 1994).

The life span of the cell subsets is also an important factor in the generation and maintenance of memory. There is an increasing amount of evidence demonstrating that memory cells are short-lived (particularly in the absence of antigen) and divide more rapidly than naive cells (Gray and Matzinger, 1991; Sansom et al. 1993; Zinkernagel et al. 1993). Bromo deoxyuridine (BrdU) incorporation studies in mice and the analysis of human cells in irradiated patients are examples of such evidence implying that memory lies within a population of short-lived cells requiring continual stimulation for the maintenance of memory (Michie et al. 1992; Tough and Sprent, 1994).

The phenotypic switch from high to low MW isoforms is accompanied by changes in various adhesion molecules that indicate differences in circulatory patterns and the ability to home to the site of the initial activation. High endothelial venules (HEV) bind to L-selectin, a molecule strongly expressed by RA^+ cells and is down-regulated during cell activation while other molecules such as CD44 and CD54 are up-regulated. For example, RA^+ T cells that switch to RO^+ T cells in the skin preferentially home to this site as memory cells with the aid of increased cutaneous lymphocyte - associated antigen (CLA) and E-selectin (Picker et al. 1993a; Picker et al. 1993b). The circulatory patterns of CD45R subsets reveal that RA^+ cells cross the high endothelial venules to enter lymph nodes and leave through the efferent lymphatic duct. RO^+ cells leave the blood and enter the tissues through the endothelium and drain into the lymph node via the afferent lymph duct (Picker and Butcher, 1992). Recirculation in this manner would ensure that RO^+ 'memory' cells home to the tissues where they are most likely to encounter a specified antigen. For RA^+ 'naive' cells rapid recirculation from blood to lymph would increase the possibility of a cell encountering antigen on specialised dendritic cells.

Evidence that RO^+ cells are not memory cells.

A switch from high to low MW isoforms (RA to RO in humans), could simply indicate the activation state of a cell. A number of pieces of evidence believed to support the idea that RO^+ cells are memory cells could also be used to argue against this suggestion.

For example, increased levels of second messengers and enhanced expression of adhesion / co-stimulatory molecules all indicate recent activation. The change in isoform in itself is not only unstable in some circumstances but can occur in the apparent absence of antigen (Rothstein et al. 1991). Thymocytes switch from RA to RO prior to undergoing thymic selection (Yang and Bell, 1992; Bell and Sparshott, 1990). Antigens have not been detected during this process and RO is continually expressed until immediately before exit from the thymus. Thymocytes gain expression of RA and enter the periphery as mature, naive cells (Gillitzer and Pilarski, 1990; Deans et al. 1991; Serra et al. 1988). Clearly, thymocytes must undergo some degree of activation during positive and negative selection, hence the switch in CD45 isoform. This does not mean that all RO^+ thymocytes are memory cells.

The conversion to RO following activation can be reversed (see section 1-9) which is strong evidence to support the idea that changes in CD45 isoform are indicative of cell activation and not a switch from naive to memory. The induction of RA $\rightarrow$RO switching in the absence of TCR signalling (mitogenic stimulation) also indicates the activation state of the cell and not the conversion from naive to memory. Exposure of humans to radiation would effectively result in a similar situation to a thymectomy in mice with respect to the production of mature T cells. Analysis of CD45R populations in the periphery following irradiation did not result in a significant decrease in naive cells. Also, RA^+ T cell numbers do not decrease in humans after a thymectomy (P. Beverley, personal communication). Clearly, such a switch occurs during activation and potential memory cells may exist in this population. More evidence is required to identify which effector cells become memory cells and how this occurs.

T CELL ALLOREACTIVITY.

T cells respond to soluble antigen when it is processed and presented by host APC in complex with self MHC molecules. The T cell is only able to recognise soluble antigen in this manner. Responses to tissues transplanted from one individual to an unrelated recipient of the same species are different. Allorecognition involves the host immune system recognising and responding to donor tissue.

Since T lymphocytes can only respond to foreign antigens in complex with MHC molecules, one of two processes is occurring; either the host responds directly to foreign MHC molecules expressed on the transplanted graft or to foreign antigens complexed in alloMHC (Matzinger and Bevan, 1977). The issue of donor tissue processed by host APC and presented (MHC and minor histocompatibility antigens) in the context of self MHC is a particular case of the normal process known in transplantation as the indirect MLR. If lymphocytes from donors of the same species with differing MHC types are cultured together, T cells from either donor will proliferate in response to the foreign MHC, alloMHC (Merkenschlager et al. 1988; Morimoto et al. 1985b). This is an *in vitro* model of the response observed in graft rejection and is termed a mixed lymphocyte reaction (MLR) (Bach et al. 1976). In addition to being a method of predicting graft rejection, the MLR is a valuable tool in analysing the events involved in T cell activation and the role of the MHC molecule.

A large proportion of naive T cells can recognise and respond to alloantigen, resulting in vigorous proliferation and rapid graft rejection. Between 1-10% of T cells alloreactive. This is 100 fold higher than the number of T cells specific for any soluble antigen in primed individuals but is similar to the repertoire reacting with superantigens (Lindahl and Wilson, 1977). There are striking differences between soluble and allo- antigens, one of which is the type of CD45R subset involved. RO^+ T cells are solely responsible for proliferation against soluble recall antigens yet both RO^+ and RA^+ T cells will respond to alloantigen expressed on foreign APC with similar kinetics and frequency (Merkenschlager et al. 1988; Morimoto et al. 1985b).

An early hypothesis, proposed by Matzinger and Bevan, to explain why such a high frequency of alloreactive T cells existed, involved the presentation of a variety of native peptides by foreign MHC molecules on the surface of alloAPC to both $CD4^+$ and $CD8^+$ cells. The alloMHC in complex with a self peptide would form complexes host T cells recognise and respond to. Proliferation against the MHC molecule itself would occur, not necessarily relying on the specificity of the peptide (Matzinger and Bevan, 1977). This has been demonstrated for MHC class I and II molecules and is termed the 'direct pathway' of allorecognition (Fig. 1-7A). The indirect pathway involved the presentation of alloantigens by self MHC and was proposed a few years later.

The alloMHC are shed by the donor organ cells and processed by self APC as foreign peptides (Wecker and Auchincloss, 1992). This pathway would predominantly employ $CD4^+$ T helper cells as the peptides are exogenous (Fig. 1-7B).

The amalgamation of both pathways would result in a higher frequency of precursor T cells than would normally occur in a T cell response to soluble antigen. The direct pathway is capable of stimulating both $CD4^+$ and $CD8^+$ T cells whereas the indirect pathway involves the presentation of exogenous antigen and therefore requires MHC class II APCs. Dendritic cells are important in alloresponses. Rat renal grafts depleted of donor DCs are retained indefinitely and tolerance can only be broken by the infusion of donor DCs .

It is well documented that MHC class II^+ APC are the main stimulators in a primary immune response against alloantigen (Katz and Sant, 1994). Via *et al* demonstrated the ability of alloAPCs to stimulate both $CD4^+$ and $CD8^+$ T cells, this is obviously an example of the direct pathway. The removal of alloAPCs from the MLR abolished proliferation by $CD8^+$ cells but not $CD4^+$, indicating that $CD4^+$ T cells were capable of using the indirect pathway but $CD8^+$ cells were not (Via et al. 1990; Muluk et al. 1991). Alloreactivity is a complex response involving higher frequencies of T cells and alternative APCs that are not employed in responses to conventional antigen.

This thesis examines the minimal activation requirements for stimulation of CD45R subsets based on alloantigen responses. Since the role of accessory molecules in T cell activation is complicated by a background of multiple interactions between the T cell and the APC, the use of transfected cell lines permits carefully controlled studies of T cell responses.

The murine fibroblast cell line, DAP.3 was transfected with cDNA's encoding the human MHC class II molecule, HLA-DR1 with either of the accessory molecules CD54 (ICAM-1) or CD58 (LFA-3) and used as allostimulators for purified CD45R T cell subsets.

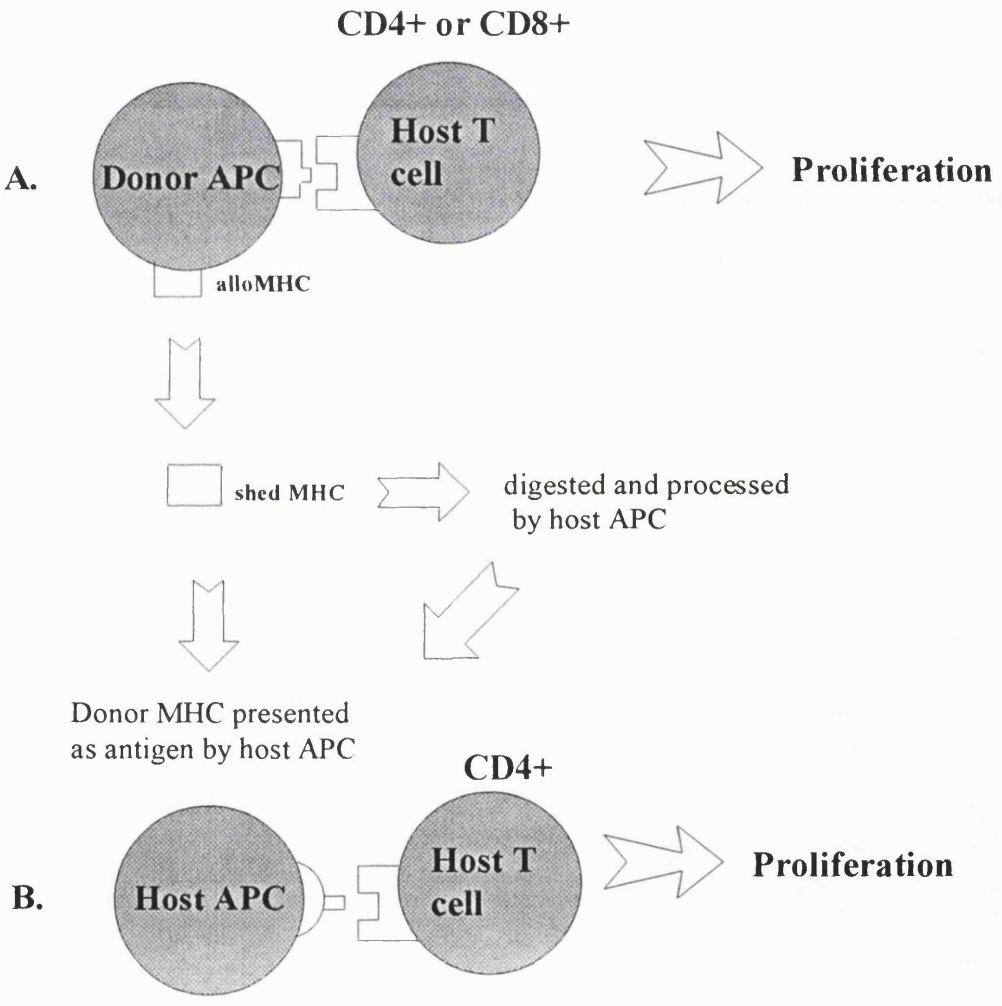
Adhesion molecules are believed to be the initial contacts between a T cell and an APC. These molecules form close contacts between the cells prior to TCR engagement. CD2 and CD11a/CD18 are two of the main adhesion molecules expressed by a T cell.

Figure 1-7. Pathways of alloantigen presentation to host T cells.

A.) Direct pathway- presentation of foreign or native peptides by donor MHC.

B.) Indirect pathway- the processing and presentation of alloMHC by the host MHC to $CD4^+$ T cells.

Fig.1-7: Pathways of alloactivation



Interaction with their ligands CD58 and CD54 respectively are thought to transduce intracellular co-stimulatory signals as well as forming strong contacts between the cells. For this reason, CD54 and CD58 were selected as the accessory signals on the TFCL.

It was predicted that RO^+ T cells should respond more readily to the TFCL and that extra triggering requirements (such as cytokines or co-stimulatory signals) would be required for RA^+ T cells.

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CHAPTER 2-METHODS.

LYMPHOCYTE SEPARATION

Isolation of peripheral blood mononuclear cells.

Heparinised venous blood was obtained from normal healthy adults. All blood was kindly donated by healthy volunteers in the Department of Clinical Immunology, RFHSM and polycythaemic patients under the care of Prof. Hoffbrand (Dept. Haematology, RFHSM). Polycythaemic patients undergoing routine venesection were included in this study as a normal source of PBMC providing venesection was the only treatment they were undergoing at that time. All blood donors were HLA-DR1 negative and were stained using the definitive activation panel to eliminate donors with raised levels of activation markers at the onset of the separation procedure (see Table 2-II and Appendix 2-I). Mononuclear cells were separated as previously described (Boyum, 1974). Briefly, whole blood was separated by density gradient centrifugation over Lymphoprep at room temperature. The cells recovered from the interface were washed three times in Hanks balanced salt solution (HBSS) supplemented with 5% heat inactivated foetal calf serum (FCS) and resuspended in RPMI 1640 supplemented with 10% FCS, 2mM L-glutamine, 50μg/ml streptomycin and 50U/ml penicillin (complete culture medium) at a concentration of $1x10^6$ cells/ml.

Monocyte Depletion

Monocytes were removed using a G10 sephadex column (see Section 2.1.3). This procedure was used as an effective method for removing adherent cells if they were not required for experimental use (Ly and Mishell, 1974). Cells were passed down the column and washed thoroughly in HBSS before being resuspended in complete culture medium at a concentration of $1x10^6$ cells/ml.

Preparation of the G-10 column

Sephadex G10 was swollen for 1hr at 60°C in PBS and washed thoroughly in PBS three times before finally being resuspended in fresh PBS 1.3 times the bed volume. The mixture was then autoclaved and aliquoted into 50ml volumes for future use.

A 50ml sterile syringe with a wide bore outlet was attached to a butterfly needle and clamped to a ring-stand. A small quantity of glass wool soaked in Sephadex was used to plug the syringe outlet to prevent the Sephadex from running out of the column but still allowing HBSS to pass through. Clean, acid washed glass beads (10g) were poured into the syringe barrel in 10mls of HBSS supplemented with 5% FCS, 40mls of Sephadex was then added and allowed to settle. The column was washed with 200mls of 5% FCS-HBSS before cell suspensions were passed through at a concentration of $1x10^8$ cells/ml over 20 mins. Finally, harvested cells were washed twice in HBSS and resuspended in complete culture medium at a concentration of $1x10^6$cells/ml.

Isolation of monocytes

Monocytes were purified by plastic adherence to culture flasks coated with 2% gelatin solution. Culture flasks were prepared as follows; $75cm^3$ culture flasks were incubated with 10mls of gelatin solution for 1hr at 37°C, followed by an overnight incubation at 55°C in a drying oven. Flasks could then be stored at room temperature until required and re-hydrated with complete culture medium for 1hr at 37°C when necessary. Mononuclear cells ($2x10^7$cells/ml) were incubated in the gelatin coated flasks for 90mins at 37°C, 5% CO_2. Non-adherent cells were removed by washing with HBSS. Adherent cells incubated with a chilled 1:1 solution of 10mM disodium EDTA in PBS and complete culture medium for a further 10mins at 4°C and washed thoroughly in HBSS supplemented with 5% FCS before finally being resuspended in complete culture medium at a concentration of $1x10^6$/ml. Monocytes isolated in this manner were >90% $CD14^+$ assessed by FACScan® analysis (Becton Dickinson, UK). Purified cells were kept at 4°C until required to prevent re-adherence.

Purification of CD45R subsets.

CD45R T cell subsets were separated by immunomagnetic bead selection from Lymphoprep separated or monocyte depleted PBMC. Only negatively selected cells were used in assays to avoid alteration in lymphocyte function caused by MAb bound to membrane proteins. PBMC were incubated with a cocktail of MAbs, all used at optimal concentration.

The cocktail always included MAbs against HLA-DR to remove B cells and activated T cells, CD16 to remove NK cells together with either CD4 or CD8 and CD45RA or CD45RO (see Table 2-II). Selected cells were removed by adding either GαM IgM and /or IgG magnetic beads suspended in serum free HBSS. Incubation with magnetic beads was repeated twice to produce highly purified subsets. Populations were >95% pure as determined by immunofluorescent staining (see Appendix 2-I and 2-IV).

Preparation of T cells by E rosette formation.

Human T cells express CD2, a co-stimulatory molecule that binds to sheep red blood cells as well as ligands expressed on host APC. Ten mls of sheep red blood cells (SRBC) in an anti-coagulant, Alsevers solution, were washed three times in HBSS supplemented with 5% FCS by centrifugation and resuspended in 10mls of serum free culture medium with 400μl of 1unit/ml neuraminidase. Following a 1hr incubation at 37°C, SRBC were washed twice in serum free HBSS before finally being resuspended in 10mls complete culture medium.

Whole PBMC in complete culture medium at a concentration of $1x10^6$ cells/ml were incubated with 1ml of neuraminidase treated SRBC and 2mls of heat inactivated FCS for 30mins at 37°C. The mixture was pelleted by centrifugation and incubated for a further 2hrs on ice. The cell pellet was gently resuspended in the same medium and separated by density-gradient centrifugation. Non-T cells at the interface were discarded and the T cell /SRBC rosettes in the cell pellet were lysed with Hoffman's lysis buffer until the solution became transparent and washed three times in HBSS supplemented with 5% FCS. Purified E RFC^+ T cells were finally suspended in complete culture medium at a concentration of $1x10^6$cells/ml.

Fixation of MLR stimulator cells.

Two techniques of fixation were employed to determine the optimal method appropriate for one way MLRs. TFCL, LCL and autologous monocytes in complete RPMI at a concentration of $1x10^6$ cells/ml, were treated with either mitomycin C (MMC) or fixed with 1% paraformaldehyde solution.

For MMC treatment, stimulator cells were incubated for 30mins at 37°C with 1μg/ml of MMC, then washed in HBSS supplemented with 5% FCS three times before being resuspended in complete culture medium. For paraformaldehyde fixation, stimulator cells were pelleted by centrifugation and incubated with 1ml of 1% paraformaldehyde solution for 1min at 37°C, washed in HBSS and resuspended in complete culture medium at the required concentration.

Determination of cell viability.

Trypan blue at a final concentration of 0.08% in physiological saline was used to determine viability of the purified T cell subsets and cell lines. Cell concentrations were counted by standard haematological techniques using a Neubauer counting chamber, light microscopy and a 1:1 mixture of Trypan blue and cell suspension.

IMMUNOPHENOTYPING.

Monoclonal antibodies and immunofluorescent staining.

Monoclonal antibodies (Table 2-I) were used at optimal concentrations. Indirect, dual fluorescent staining involved a two stage procedure, $3x10^5$cells were prepared as described in Section 2.1.3 and incubated with the required unconjugated MAbs for 10 mins at room temperature followed by six washes in phosphate buffered saline supplemented with albumin (PBSA). Cells were then incubated with optimal concentrations of PE conjugated GαM IgM and FITC conjugated GαM IgG for a further 10 mins. Finally, cells were washed and fixed with 1% paraformaldehyde. Negative controls were normal mouse serum, APC5 and CD19. Samples were analysed on FACScan as previously described. Briefly, T cells were separated from non-T cells on the PE channel and positive from negative T cell antigen expression using the FITC channel. Positive and negative controls were used to set gates to determine positive antigen expression (Amlot, 1996) TCR αβ expression was used to to define the T cell population.

CYTOKINES AND OTHER REAGENTS.

Lyophilized IL-1β, IL-2, IL-4 & IL-6 were reconstituted with 100μl of 5% acetic acid solution, further dilutions were made in phosphate buffered solution and stored at -20°C.

All cytokines were a kind gift from Dr.B. Richardson, Sandoz Ltd, Basle, Switzerland. Human recombinant IL-2 was titrated on an IL-2 dependent cell line, CTLL. Briefly, $1x10^4$ cells/ml were washed in serum free medium and resuspended in complete culture medium in flat-bottomed 96 well plates with increasing concentrations of IL-2. Proliferation was assessed by [^{3}H]TdR following a 4hr pulse. IL-1β, IL-4, IL-6 and IFNγ were titrated using CD3 and LCL stimulated PBMC in 3 and 5 day assays respectively. The mitogen Phytohaemoglutanin (PHA) was used at a final concentration of 1μg/ml in T cell proliferation assays as a control.

CELL CULTURE

The human EBV transformed lymphoblastoid cell line (LCL), KAS116, is an HLA-DR1$^+$ homozygous cell line and was obtained from the 10th International Histocompatibility Workshop and cultured in complete culture medium (see Table 7-I for details of antigen expression). The mouse fibroblast cell line DAP.3 (FCL), was transfected with genes encoding human HLA-DR1 and either human CD54 (ICAM-1) or CD58 (LFA-3) as previously described (Lechler et al. 1988; Greenlaw et al. 1992). The FCL was cultured in complete culture medium, CD54$^+$TFCL and CD58$^+$TFCL were cultured in complete medium supplemented with geneticin (200μg/ml; ICN Flow UK). Complete medium containing MXH was used to culture DR1$^+$TFCL and DR11$^+$TFCL. Double transfectants (DR1$^+$CD54$^+$TFCL and DR1$^+$CD58$^+$TFCL) were maintained in complete medium with both MXH and geneticin (see Table 2-II). MXH consists of Mycophenolic acid, xanthine and hypo-xanthine (see Appendix 2-II for details).

Table 2-I: Monoclonal antibodies and their sources.

Name	MAb	Subclass	Source
TCRαβ	T10B9	IgM	Prof. J Thompson
CD2	RFT11	IgG_{2a}	RFHSM
CD3	MEM57	IgG_{2a}	Biogenesis
CD4	RFT4	IgG_1	RFHSM
CD5	RFT1	IgM & IgG_1	RFHSM
CD8	RFT8	IgM & IgG_1	RFHSM
CD11a	DR9 5E9	IgG_1	RFHSM
CD14	UCHM1	IgG_{2a}	RFHSM
CD16	Leu 11b	IgM	Prof. J Thompson
CD19	RFB9	IgG_1	RFHSM
CD21	RFB6	IgG_1	RFHSM
CD25	RFT5γ2a	IgG_{2a}	RFHSM
CD26	TA1	IgG_1	Coulter
CD28	9.3	IgG_{2a}	E Clarke
CD29	Integrin β1	IgG_1	Becton Dickenson
CD38	RFT10	IgG_1	RFHSM
CD45RA	SN130	IgG_1	RFHSM
CD45RB	PD7	IgG_1	Becton Dickenson
CD45RO	UCHL1	IgG_{2a}	P. Beverley
CD49d	VLA-4	IgG_1	Becton Dickenson
CD50	ICAM-2	IgG_1	Serotech
CD54	ICAM-1	IgG_1	Serotech
CD58	LFA-3	IgG_{2a}	Immunotech
CD62L	L-selectin	IgG_{2a}	Becton Dickenson
CD69	AIM	IgG_{2b}	Serotech
CD80	B7-1	IgG_1	Becton Dickenson
CD102	ICAM-3	IgG_1	C. Buckley
HLA-DR	RFDR2	IgG_{2a}	RFHSM
NMS	Normal Mouse serum		Balb C mice
control	APC5	IgG_{2a}	M. Parkhouse
IgG-FITC			Southern Biotechnology
IgM-PE			Southern Biotechnology

RFHSM-Generated in the Department of Immunology, Royal Free Hospital School of Medicine

Cells were cultured at 37°C in 5% CO_2 and passaged every 3 to 4 days by trypsinisation when necessary. Briefly, the existing culture medium was poured off into a sterile centrifuge tube and spun to a pellet. Adherent cells in a $75cm^3$ culture flask were washed in serum free HBSS and incubated with 5ml of trypsin solution and gently dislodged. The harvested cells were washed thoroughly in HBSS supplemented with 5% FCS, pooled with the non-adherent pellet and resuspended in the specified selective medium at a concentration of $1x10^5$cells/ml. All cell lines were found to be free of mycoplasma contamination on repeated testing following culture in antibiotic free medium for three weeks. Mycoplasma testing was performed using the ICN kit employing the Hoechst stain.

Table 2-II: Cell lines used as stimulator populations.

Transfection	**Cell Line**	**Identification**
Control	DAP.3	FCL
HLA-DR1	531	$DR1^+$TFCL
HLA-DR11	RGT1	$DR11^+$TFCL
CD58 (LFA3)	LFA3	$CD58^+$TFCL
CD54 (ICAM-1)	ICAM1	$CD54^+$TFCL
HLA-DR1 + CD58	531/LFA3	$DR1^+CD58^+$TFCL
HLA-DR1 + CD54	531/ICAM1	$DR1^+CD54^+$TFCL
None	KAS116*	$DR1^+$LCL

*Homozygous $DR1^+$ B lymphoblastoid cell line (LCL), not transfected.
All stimulator cell cultures were regularly checked using immunofluorescentstaining to ensure expression of HLA-DR and accessory molecules (see Table 2-III).

PROLIFERATION ASSAYS.

Mixed Lymphocyte Reaction (MLR)

Proliferation assays were performed using $1x10^5$ responder cells cultured in a 96-well flat bottomed plate for 5 days with $5x10^4$ MMC treated $DR1^+$LCL, TFCL or $1x10^4$ purified monocytes in a final volume of 200µl/ well. Plates were incubated at 37°C, 5% CO_2 for 6 days and pulsed (50µl/well) with a [^{3}H]TdR solution (50 Ci/ml, 1mCi/mmole specific activity) for the last 18hrs of the culture.

Cells were harvested onto glass fibre filters and β-emission was assessed by liquid scintillation counting. Each sample was performed in triplicate.

Proliferation is represented as a stimulation index, $SI = \frac{[^3H]TdR \text{ test - background}}{[^3H]TdR \text{ control - background}}$

Control = responder cells alone, an SI>2.5 was considered a positive response.

Priming assays.

Whole PBMC were incubated in a primary MLR for 5 days with either the $DR1^+$LCL or the $DR1^+$TFCL in a $25cm^3$ culture flask. On day 6, cells were washed in HBSS supplemented with 5% FCS and resuspended in fresh culture medium at a concentration of $1x10^6$/ml for a further 24hrs. On day 7, cells were rechallenged for a further 5 days by either of the stimuli used in the primary MLR. Proliferation was assessed by a 4hr pulse with [^{3}H] TdR on days 3, 5, 6, 7, 9, and 11. Viability was assessed on the same days by trypan blue exclusion.

Co-stimulation assays.

In experiments with CD28 MAb, culture wells were incubated for at least an hour at 4 °C with 100μl (1μg/ml) GαM IgG, washed in sterile PBS, followed by 1hr at 4°C with CD28 MAb (9.3, 1μg/ml). After extensive washing of the wells with sterile PBS, cells were added and incubated at 37°C, 5% CO_2 for 3 days. Proliferation was determined by [^{3}H]TdR incorporation.

Statistical analysis

Multiple comparisons between sample groups were analysed by ANOVA using either the Dunnett's test or the Tukey-Kramer test. All analyses were performed using the statistical program Instat2.

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Table 2-III: Expression of HA-DR and accessory molecules on various APCs.

APC	Control (NMS)	HLA-DR	CD54	CD58
FCL	21	17	17	26
$DR1^+$TFCL	16	447	21	18
$CD54^+$TFCL	17	22	120	12
$CD58^+$TFCL	16	25	15	176
$DR1^+CD54^+$TFCL	19	440	172	19
$DR1^+CD58^+$TFCL	14	555	28	189
$DR1^+$LCL	19	1221	386	364
*Autol. Monocytes	18	653	299	268

* Purified monocytes >95% $CD14^+$. For separation procedure, see Section 2.1.4. All other APC types are homozygous populations and were gated on forward and side scatter. The numbers represent the MFI of 2000 gated events. The above table shows one of five experiments carried out in conjunction with studies described in Chapter 6.

CHAPTER 3

RESPONSES BY T CELL SUBSETS TO TFCL.

INTRODUCTION

RA^+ and RO^+ T cells differ in their ability to respond to soluble recall antigens such as tetanus toxoid. Responses to this type of antigen lie exclusively with the RO^+ subset (Wallace and Beverley 1990; Smith et al. 1986; Sanders et al. 1988), whereas responses to alloantigens are by both populations with similar frequency and kinetics (Sanders et al. 1988; Merkenschlager et al 1988).

This project attempts to identify differences in responses by subsets of CD45R T cells to alloantigen. Many studies have been unable to differentiate between these two populations using alloantigen presented on allogeneic monocytes see Table 1-II (Akbar et al 1988; Akbar et al. 1989, Sanders et al. 1988; Merkenschlager et al 1988). RO^+ T cells are thought to be more readily activated than RA^+ T cells since they express pre-formed signalling complexes with CD4, CD45 and TCR/CD3 (Dianzani et al. 1992; Janeway, Jr. 1992). In addition, levels of second messengers such as DAG and IP_3 are elevated (Robinson et al. 1993). Both of these pieces of evidence indicate that RO^+ T cells are more prepared for proliferation or triggering than their RA^+ counterparts.

CD45R populations also vary in their expression of co-stimulatory molecules (see Table 1-II). In particular, RA^+ T cells express CD2 and LFA-1 at a lower density compared to RO^+ T cells (Sanders et al. 1988). It therefore follows that RO^+ T cells will be more responsive to TFCL stimulation involving either of these pathways in allogeneic activation.

The use of transfected cell lines in studies such as this has many advantages. Firstly, the type of alloantigen used as the stimulator is consistent compared with the variation in human allogeneic APCs even when homozygous HLA stimulating cells are selected. Secondly, transfected cells allow control of the molecular interactions between the T cell and the APC. Ideally, the only interactions between the T cell and APC should be through molecules transfected into the stimulator cells and their ligands. Even without the limitation of transfected molecules the background remains identical between experiments.

The generation of MHC class II restricted cell lines has been a valuable tool in the study of the relationship between T cells and APCs. For example, T cell proliferation against PHA, CD3 MAb and superantigens can occur in the presence of HLA-DR TFCL (Altmann et al. 1989). In most cases, the level of HLA-DR expression is critical when HLA-DR transfected cells are the stimulator population (Altmann et al. 1989; Merkenschlager, 1991; Fischer et al. 1992).

CHO cells co-transfected with HLA-DR4 and either CD80, CD54 or CD58 identified differences in adherence between RA^+ and RO^+ T cells. Adherence to $DR4^+$ $CD58^+$ TFCL by RO^+ T cells was much stronger than by RA^+ T cells. The kinetics of both CD45 populations were very similar for adherence to $DR4^+CD58^+$TFCL and $DR4^+CD80^+$TFCL (Parra et al. 1993). It would be interesting to see if these differences were extended to activation of CD45R populations using similar APC.

Studies using L cells transfected with HLA-DR2 and CD54 were capable of inducing allo-proliferation. The expression of both molecules was essential as either DR2 alone or CD54 alone had no effect (Altmann et al. 1989). Also, if T cells were stimulated with CD3 MAb in the presence of CHO cells transfected with either CD80 or CD58, only CD80 was able to induce proliferation (Sanson et al. 1993). If SEB was used in place of CD3 MAb, either co-stimulus induced activation providing HLA-DR and the co-stimulus were on the same cell surface. If these two signals were on separate cells, CD80 but not CD58 would induce proliferation.

Finally, initial experiments by Greenlaw *et al* using the TFCL described in Chapter 2, showed that HLA-DR1 co-transfected with GPI-linked CD58 could function as an APC for $HLA\text{-}DR1^-$ T cell clones and that both the TCR signal and the co-stimulus must be expressed on the same cell surface. Interestingly, they showed that the double transfectants ($DR1^+CD58^+$ or $DR1^+$ $CD54^+$) induced higher levels of proliferation by RO^+ than RA^+ T cells (Greenlaw et al. 1992).

A number of preliminary experiments were necessary to establish baseline responses of isolated human T cells in the presence of FCL. Firstly, since the level of HLA-DR expression has been shown to be vital in previous studies, levels of HLA-DR1 expression were established for all stimulator populations utilised and were repeatedly monitored to ensure that APCs maintained comparable levels of MHC class II expression for each experiment (see Section 2-4). Secondly, responder to stimulator ratios were titrated to establish their optimal proportions. Thirdly, separation and fixation methods were examined.

Fourthly, since the CD2-CD58 pathway was one of the accessory molecule pairs being studied, the effect of T cell purification by E-rosette formation was assessed. Positive and negative selection procedures were compared to ensure that T cell populations were not affected by the techniques used. Finally, all studies on alloantigen specific T cells were carried out in one-way MLRs. Stimulator cells must therefore be fixed to prevent cell division whilst maintaining the ability to present antigen and induce activation. The experiments described in this Chapter established the protocol used in the subsequent experiments.

EXPERIMENTAL DESIGN

All experiments were carried out in triplicate and analysed by statistical methods described in each case.

RESULTS

Titration of stimulator and responder cells.

Whole PBMC or CD45R T cell subsets from three HLA-DR1 negative individuals were stimulated in an MLR for 6 days with increasing numbers of stimulator cells (Fig.3-1A to C). Stimulator cells were titrated against a constant number of responder cells ($1x10^5$) and proliferation was assessed by [^{3}H]TdR uptake.

Optimal stimulation by $DR1^+$ TFCL was achieved using a 1:10 stimulator responder ratio (Fig 3.1A) for whole PBMC. Any further increase in the number of stimulator cells resulted in a rapid decline in proliferation. CD45R T cell subsets demonstrated reduced proliferation levels in comparison to whole PBMC and did not show a particularly distinct optimal response. $DR1^+CD58^+$ and $DR1^+$ $CD54^+$ TFCL induced proliferation by PBMC and CD45R subsets and all peaked at $5x10^4$ stimulator cells, a ratio of 1:2 responder to stimulator cells (Fig. 3.1B). In the case of $DR1^+$ LCL stimulation (Fig 3.1C), $5x10^5$ induced peak proliferation with whole PBMC and between $1x10^5$ & $5x10^5$ stimulators were required for maximum proliferation by CD45R subsets, a ratio of 5:1. For convenience, all the TFCL were used at a concentration of $5x10^4$ (a stimulator/responder ratio of 1:2) and $DR1^+$ LCL was used at $5x10^5$.

Proliferation of an increasing number of responder cells against a constant number of stimulators; $5x10^4$ for TFCL and $5x10^5$ for LCL; results are shown in Fig. 3-2. Maximum proliferation by PBMC and CD45R subsets was induced by $DR1^+CD58^+$ TFCL at a stimulator/responder ratio of 1:1.

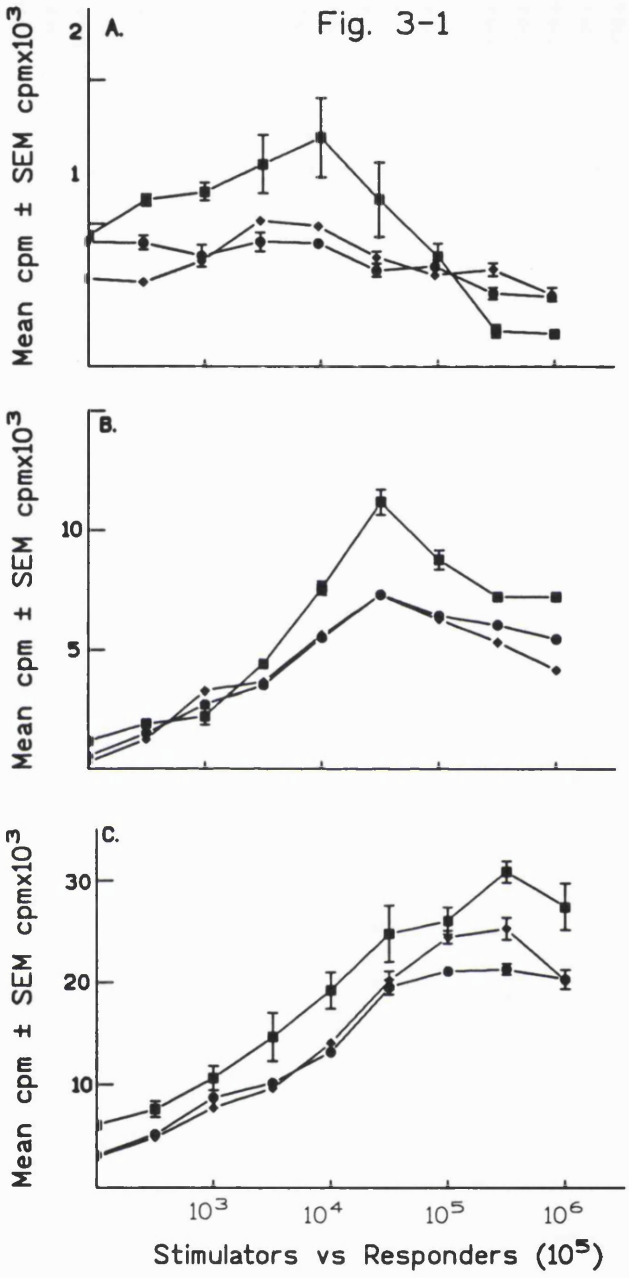

Figure 3-1: Dose response of stimulator cells.

The response to different numbers of stimulator cells by PBMC (■—■); RA^+ (●—●) or RO^+ (♦—♦) T cells. Stimulator cells were A.) $DR1^+$ TFCL; B.) $DR1^+$ $CD58^+$ TFCL or C.) $DR1^+$ LCL. $1x10^5$ responder cells were cultured in an MLR for 6 days with increasing numbers of stimulator cells. Proliferation was assessed by $[^3H]$TdR incorporation. Graphs represent one of three experiments.

Fig. 3-2

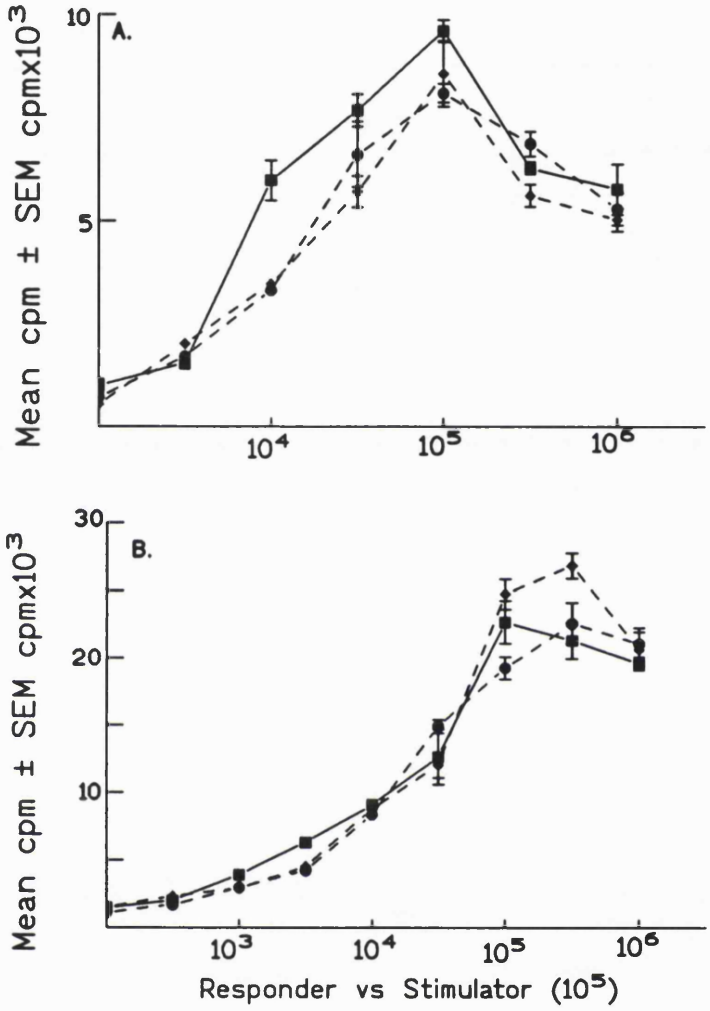

Figure 3-2: Dose response of responder cells.

The dose response of PBMC (■—■); RA^+(●—●) or RO^+ (♦—♦), T cells against $1x10^5$ stimulator cells. Stimulator cells were A.) $DR1^+CD58^+$ TFCL or B.) $DR1^+$ LCL alloAPC. Proliferation was assessed by [^{3}H]TdR uptake on day 6. Graphs represent one of three experiments.

Optimal proliferation against $DR1^+$ LCL was achieved using a 1:1 stimulator to responder ratio for whole PBMCs and 1:5 for CD45R subsets. Following the above titration studies, the responder/stimulator ratios used in all future experiments were 1:1 for TFCL and 5:1 for $DR1^+$ LCL.

In general whole PBMC responded more vigorously to all stimulator populations compared to purified CD45R subsets. $DR1^+$ LCL induced the highest proliferation in PBMC and CD45R populations and $DR1^+$ TFCL the least. $DR1^+CD58^+$ TFCL stimulation was intermediate.

Comparison between E-rosette separation and magnetic bead depletion.

The positive selection of cells using monoclonal antibodies and magnetic beads or E-rosette formation (E-RFC) as a means of T cell purification could affect the responses in the MLR. Since the CD2/CD58 pathway is one of the main accessory pathways being investigated and CD2 is also the ligand for SRBC binding in rosette formation, it was important to investigate the effects of the purification techniques employed on allogeneic T cell responses. E-RFC is a reliable method for purifying T cells from whole PBMC and only takes one round of depletion as opposed to magnetic beads which requires two or three rounds depending on the level of expression of the specified molecule and the type of antibody used. However, negative selection using magnetic beads is less likely to interfere with the activation state of the T cell during purification in comparison to E-RFC. Proliferation against FCL, TFCL or $DR1^+$ LCL by RA^+ and RO^+ subsets purified by either E-rosette formation plus magnetic bead negative depletion or magnetic beads depletion alone were compared in a primary MLR (Table 3-I).

Statistical analysis showed that cell purification using E-rosette formation had no effect on RA^+ and RO^+ T cell responses against FCL, $CD54^+$ TFCL, $CD58^+$ TFCL and $DR1^+$ TFCL. However, both RA^+ and RO^+ T cell proliferation were significantly enhanced by positive selection through E-rosette formation as a purification procedure when cells were stimulated with either $DR1^+CD54^+$ TFCL, $DR1^+CD58^+$ TFCL or $DR1^+$ LCL. Since purification using E-RFC led to a significant increase in stimulation, all subsequent experiments and depletions were performed by magnetic bead depletion alone.

Table 3-I: Comparison of magnetic bead separation and E-rosette formation on responses.

	$CD45RA^+$		$CD45RO^+$	
	Bead depletion	**E-RFC & Bead**	**Bead depletion**	**E-RFC & Bead**
FCL	500 ± 27	474 ± 42	448 ± 17	431 ± 10
$CD54^+$ TFCL	345 ± 16	324 ± 61	374 ± 15	382 ± 29
$CD58^+$ TFCL	500 ± 47	574 ± 73	448 ± 29	431 ± 17
$DR1^+$ TFCL	621 ± 42	674 ± 75	530 ± 25	559 ± 11
$DR1^+CD54^+$ TFCL	1282 ± 235	1909 ± 116*	1100 ± 133	1821 ± 177*
$DR1^+CD58^+$ TFCL	1050 ± 79	1641 ± 109*	1050 ± 79	1747 ± 181*
$DR1^+$ LCL	22445 ± 1975	26318 ± 649*	18882 ± 1743	26522 ± 575*

*$p<0.05$ using a paired t-test, comparing bead selection and E-RFC plus bead depletion. Values represent $[^3H]$TdR uptake as mean ± SEM of two HLA-DR1 negative donors.

Comparisons of mitomycin C treated stimulator cells against paraformaldehyde fixed cells.

One way MLR's were used to test the ability of TFCL to act as alloAPC for T cell subsets. To prevent cell division by the stimulator population, cells were treated with either 1 μg /ml mitomycin C (MMC) or fixed with 1% paraformaldehyde. To test which treatment was optimal for TFCL, whole PBMC were stimulated with TFCL treated by either of MMC or paraformaldehyde (Table 3-II). No significant differences in alloresponses were observed using either method (1% Paraformaldehyde or MMC), although fixation by 1% paraformaldehyde prevented fibroblast cell lines from adhering to plastic. As it was experimentally convenient to have adherent FCL or TFCL stimulators, MMC treatment was selected rather than 1% paraformaldehyde.

Table 3-II: MMC treated cells vs paraformaldehyde fixed cells.

	MMC treated cells	Fixed cells
Stimulator	Mean ± SEM	Mean ± SEM
FCL	428 ± 92	459 ± 73
$DR1^+$TFCL	593 ± 68	529 ± 39
$DR1^+CD54^+$TFCL	5251 ± 542	3283 ± 804
$DR1^+$LCL	40995 ± 1987	36828 ± 2700

Values represent [^{3}H]TdR uptake as mean ± SEM of two HLA-DR1 negative donors.

Responses by T cell subsets to transfected cell lines.

Having established the optimal conditions for performing the MLRs, examination of responses by a number of HLA-DR1 negative healthy individuals stimulated by FCL and TFCL were performed. Whole PBMC and negatively purified CD45R T cell subsets from normal, healthy HLA-DR1 negative donors were stimulated in a primary mixed lymphocyte reaction (MLR) with either MMC treated TFCL or $DR1^+$LCL. Using the resting, responder population as a baseline, stimulation indices (SI) were compared for each of the stimulator-responder pairs (Fig. 3-3 & Appendices 3-I to 3-IV). The resting, responder population was used as the control to determine the proliferative effect, if any, the control cell line FCL had on the responder population.

In general, the magnitudes represented as mean SI were between 1.5 to 2 fold greater for PBMC than for either of the CD45R subsets when stimulated with $DR1^+$ TFCL or double TFCL. The SI and percentage of PBMC responders was approximately the same for FCL, $DR11^+$ TFCL, $CD54^+$ TFCL, $CD58^+$ TFCL or $DR1^+$ TFCL (Table III).

When HLA-DR1 was co-expressed with either CD54 or CD58, the percentage of responders doubled for all cell populations tested. Actual SI values increased between 4 and 8 fold. These were the only responses by PBMC and CD45R populations against TFCL that were statistically significant when compared to FCL ($p<0.05$).

The levels of SI in PBMC or CD45R subsets stimulated by FCL, $DR1^+$ TFCL, $DR11^+$ TFCL, $CD54^+$ TFCL or $CD58^+$ TFCL were much lower than $DR1^+CD54^+$TFCL or $DR1^+CD58^+$ TFCL. No differences between $DR1^+CD54^+$ TFCL or $DR1^+CD58^+$ TFCL were observed for any of the responder populations tested.

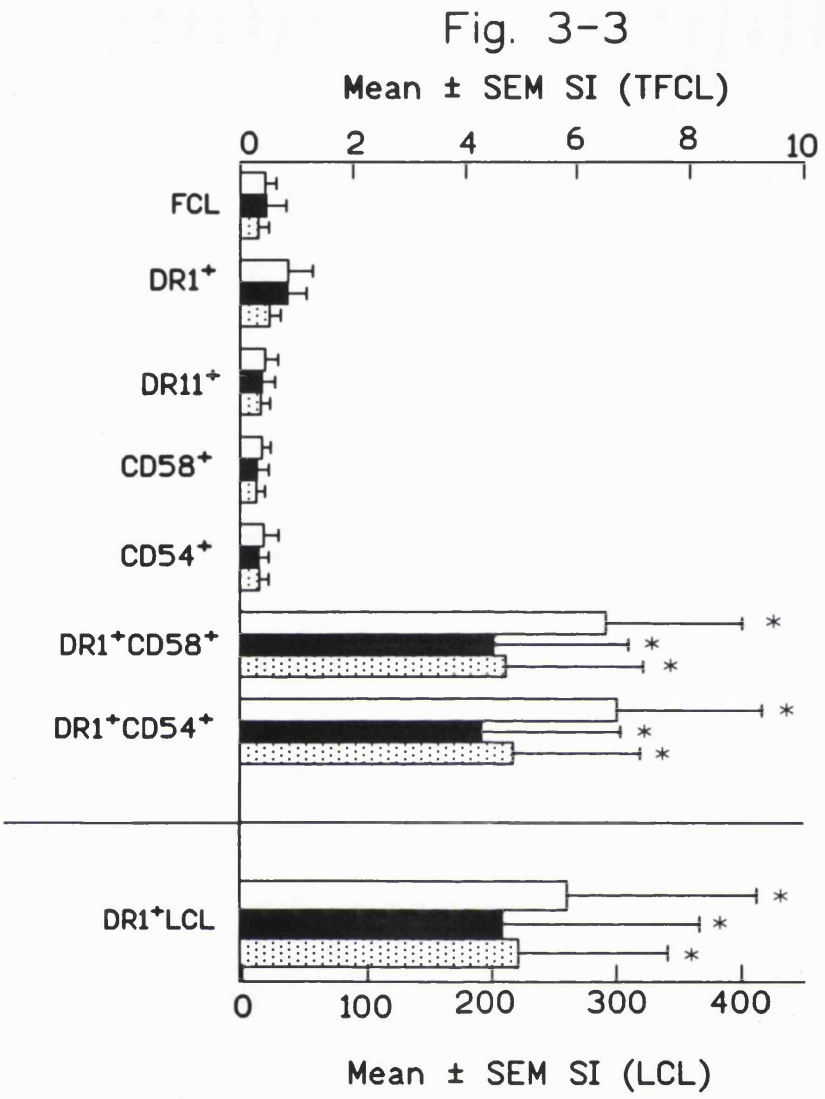

Figure 3-3: The responses by PBMC, RA^+ and RO^+ T cells to TFCL.

Responses by PBMC (open bars), RA^+ (solid bars) and RO^+ (speckled bars) against TFCL (left hand axis) or $DR1^+$ LCL (right hand axis). Bars represent the mean SI ± SEM for thirty HLA-DR1 negative donors for PBMC and fifteen individuals for CD45R subsets. SI was calculated using resting responder cells as controls.

* $p<0.05$ when sample subsets are compared to the control cell line FCL by ANOVA using Dunnetts post-test.

Generally, SI levels were lower for RO^+ than RA^+ populations against $DR1^+$ TFCL. Responses against all other cell lines were comparable. All donors responded to the $DR1^+$ LCL and were consistently greater than responses against any of the transfected cell lines.

Proliferation by CD45 T cell populations followed a similar pattern to whole PBMC. No significant difference in the average SI was observed between RA^+ and RO^+ populations.

Table 3-III: Percentage of responders with an SI ≥ 2.5.

	PBMC	$CD45RA^+$	$CD45RO^+$
	(n=30)	(n=15)	(n=15)
FCL	30 (9)	13 (2)	13 (2)
$DR1^+$TFCL	50 (15)	13 (2)	13 (2)
$CD58^+$TFCL	23 (7)	27 (4)	27 (4)
$CD54^+$TFCL	26 (8)	1 3 (2)	27 (4)
$DR1^+CD58^+$TFCL	100 (30)	80 (12)	80 (12)
$DR1^+CD54^+$TFCL	100 (30)	80 (12)	80 (12)
$DR1^+$ LCL	100 (30)	100 (15)	100 (15)

Numbers represent the percentage of donors tested, that exhibited an SI ≥ 2.5 using resting responder populations as the control. Figures in brackets are the actual number of donors responding to each stimulus (SI ≥ 2.5 when using FCL as the control).

$CD4^+$CD45R subset responses to TFCL.

To exclude the possibility that enhanced responses with LCL were due to a MHC Class I effect further purification of the CD45R subsets into $CD4^+$ RA^+ and $CD4^+$ RO^+ T cells was carried out (Bach et al. 1976; Eckels, 1990; Krensky et al. 1990).

Alloresponses against FCL, $DR1^+$ TFCL, $DR1^+CD58^+$ TFCL and $DR1^+$ LCL by $CD4^+$CD45R populations are shown in Fig.3-4. Removal of $CD8^+$ T cells and NK cells from the CD45R populations had no significant effect on proliferation, regardless of the type of stimulator cell. Low level responses to the control cell line, FCL, continued to be present.

Fig. 3-4

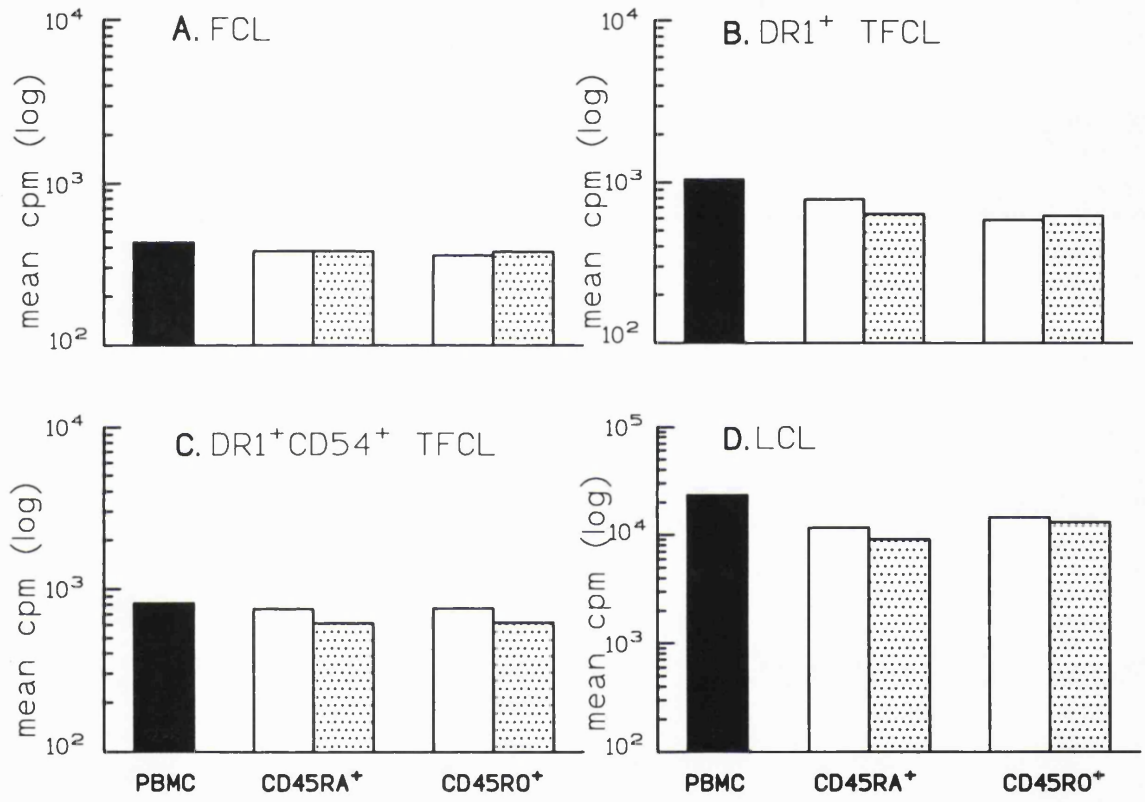

Figure 3-4: Responses by $CD4^+$ CD45R subsets.

Stimulation of PBMC (solid bars), whole CD45R subsets (open bars) and $CD4^+$ CD45R subsets (speckled bars). Stimulator populations were A.) FCL; B.) $DR1^+$ TFCL; C.) $DR1^+CD54^+$ TFCL or D.) $DR1^+$ LCL. Data represents one of four experiments.

Fig. 3-5

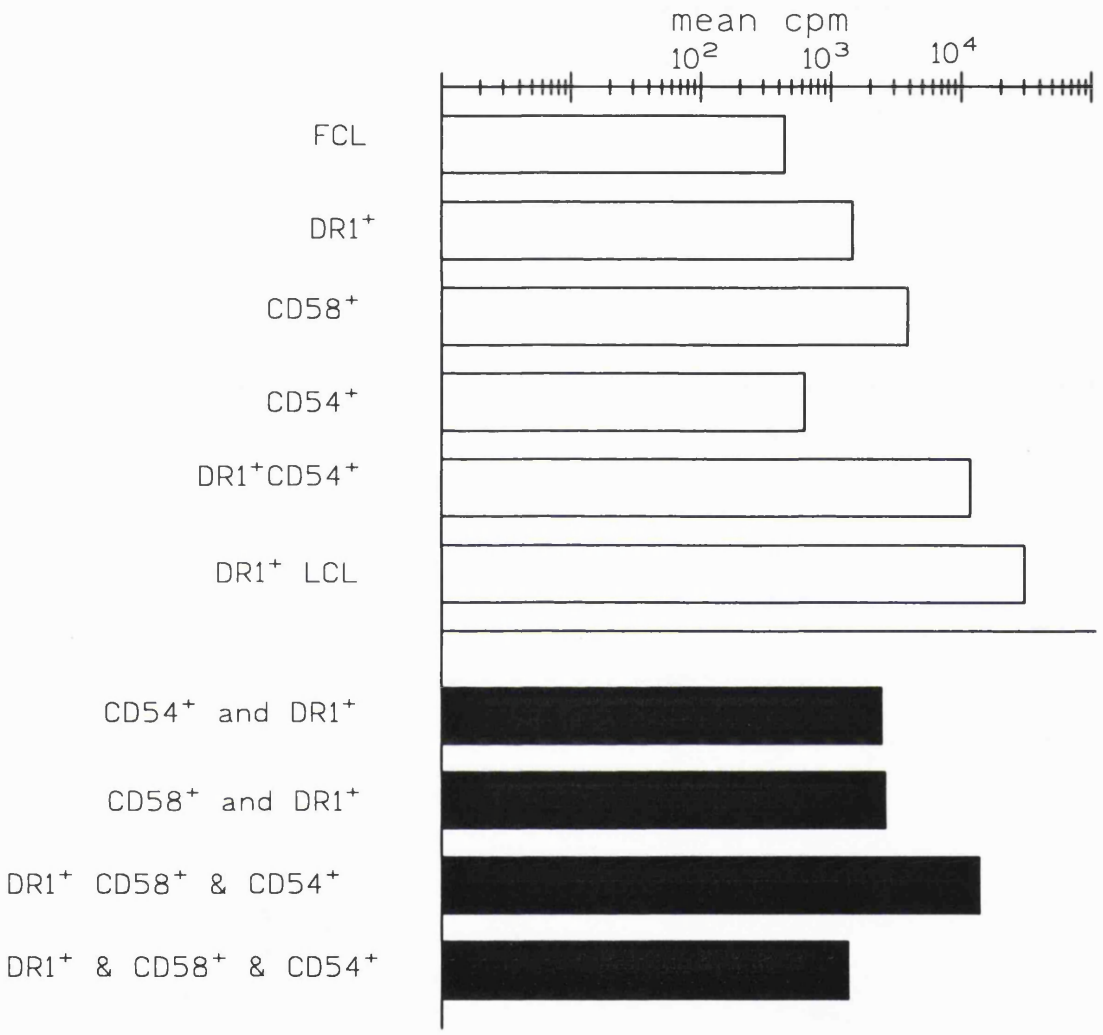

Figure 3-5: Separation of the TCR and co-stimulatory signal on TFCL.

Whole PBMC stimulated with TCR and accessory signals expressed on separate TFCL. Data represents the mean of four different experiments.

CD45R subset responses to TFCL.

To determine whether the separation of the TCR and accessory molecule signal affected the level of response by CD45R populations, whole PBMC were stimulated with TFCL, LCL or combinations of $DR1^+$ TFCL with either $CD54^+$ TFCL, $CD58^+$ TFCL or double TFCL (see Fig. 3-5). $DR1^+$ TFCL plus $CD54^+$ TFCL stimulated T cells showed levels of proliferation similar to either $CD54^+$ TFCL or $CD58^+$TFCL alone. Proliferation against $DR1^+CD54^+$ TFCL was not enhanced by the addition of $CD58^+$ TFCL by whole PBMC and CD45R populations (data not shown for CD45R populations). Similar results were observed using $DR1^+$ $CD58^+$ TFCL and $CD54^+$ TFCL.

DISCUSSION

It was necessary to titrate the stimulator / responder ratios as three of the cell lines ($CD54^+$ TFCL, $DR1^+CD54^+$ TFCL and $CD58^+$ TFCL) had not previously been used in proliferation assays of this nature. In addition, TFCL are adherent whereas the $DR1^+$ LCL are non-adherent which may have had an effect on the stimulator / responder ratio required to induce optimal T cell proliferation. The stimulator / responder ratios used in these experiments fell into the ranges obtained from similar studies (Sanson et al. 1993; Altmann et al. 1989; Greenlaw et al. 1992; Fischer et al. 1992; Parra et al. 1993).

The protocols used to purify CD45R T cell subsets can affect T cell responses to alloantigen. Separation of cells using positive selection through E-rosetting had a significant effect on RO^+ and RA^+ T cell proliferation. This effect was observed when either CD54 or CD58 were expressed on the stimulator cells. RO^+ T cells constitutively express higher densities of CD2 and LFA-1 than RA^+ cells (Sanders et al. 1988). Despite such differences in expression, positive selection of SRBC bound cells can augment activation of both CD45R T cell subsets. Previous studies also identified NK cells in the E-RFC population, which would reduce the purity of the population and require removal by positive depletion (van Kooyk et al. 1989). Stimulation by $DR1^+CD54^+$ APC does not directly involve the CD2/CD58 ligand pair although a number of studies have presented evidence to suggest that these two pathways can alter the avidity for each other and ligation of CD2 to CD58 induces a change in the activation state of LFA-1 (see Chapter 1). It has been suspected for some time that positive selection of T cells through SRBC rosetting may affect the level of activation in responder populations but no direct evidence had been demonstrated until now.

The one way MLR is an effective assay for the *in vitro* study of allogenic responses. To prevent the stimulator population from proliferating, MMC treatment was used. MMC treated TFCL and the $DR1^+$ LCL were used as stimulator populations against RA^+ and RO^+ T cells to investigate minimal signalling requirements. Unexpectedly, no statistically significant difference in alloresponses made by RA^+ and RO^+ T cells could be detected, regardless of the stimulator cell line. Alloresponses by PBMC to the TFCL were consistently greater than the CD45R subsets. Responses to the $DR1^+$ TFCL produced levels of proliferation only marginally greater than towards the control cell line, FCL, and suggested that the expression of MHC class II molecules alone on a fibroblast cell is not sufficient for T cell activation.

CD58/CD2 and CD54/CD11a-CD18 ligand binding pairs acting as co-stimulatory/adhesion pathways for T cells are well described in the literature (Springer et al. 1987; Jenkins and Johnson, 1993). The binding of CD2 and CD11a/CD18 on the T cell to their respective ligands expressed by the APC are believed to be one of the earliest contacts made between the two cells (Springer, 1990). For this reason, these ligand pairs were selected to investigate differences between RA^+ and RO^+ allostimulation. Co-transfection of either CD54 or CD58 into the TFCL induced T cell responses by PBMC and CD45R subsets and increased the percentage of responders. No differences between RA and RO T cells to doubly transfected cells was observed. This was surprising because RA^+ T cells are known to express lower densities of activation molecules such as CD54, CD2, HLA-DR, CD58 and CD25 than RO^+ T cells (see Chapter 1). It is evident that the level of accessory molecules expressed by RA^+ T cells is sufficient to induce alloresponses to function as efficiently as RO^+ T cells. Increased expression of the co-stimulatory / adhesion molecules may play a more important role in the re-circulation of T cells or against antigens presented at low levels of density.

In humans, both $CD4^+$ and $CD8^+$ T cells are able to respond to alloantigen. Responses against the control cell line FCL, were not eliminated by the removal of $CD8^+$ T cells, indicating that proliferation was not influenced by the presence of $CD8^+$ T cells. FCL induce a higher level of [^{3}H] TdR incorporation than lymphocytes alone. FCL may therefore express accessory molecules that human T cells can recognise and respond to, for example murine CD80 (B7/BB1), is expressed at high levels on FCL and has homology with human CD80 (Schwartz, 1992). Murine CD58 is also a possible candidate with a high level of homology to human CD58 (Brown et al. 1988; Sayre et al. 1987; Yagita et al. 1988).

To effectively activate T cells, signals from the APC must be co-expressed on the same cell surface. Expression of the HLA-DR and accessory signals on two separate cells abolishes the effect that accessory molecules exert on T cell activation. Experiments showed that co-expression of the two signals is vital to T cell proliferation.

Whole PBMC and CD45R populations are clearly capable of responding to cells transfected with human HLA-DR1 together with CD54 or CD58, although there was no difference between the CD45R subsets in responses to TFCL. Separation of whole PBMC into CD45R T cell subsets demonstrated similar patterns of response to whole PBMC but the magnitude of the responses was greatly reduced. Purification of PBMC into CD45R populations may remove vital signals required for maximum alloresponses against TFCL.

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CHAPTER 4.

INTERACTION BETWEEN CD45R SUBSETS IN THE MLR.

INTRODUCTION

Proliferation by $CD4^+CD45R$ T cell subsets to alloantigen show similar intensity and kinetics, resulting in large, vigorous responses (Akbar et al 1988; Sanders et al 1989; Merkenschlager et al 1988). The reduced proliferation against TFCL and LCL by CD45R subsets compared to whole PBMC suggests that either the two CD45R subsets proliferate independently and depletion of either subset reduces the response compared to whole PBMCs or the CD45R subsets collaborate during activation, causing increased proliferation against alloantigen. Interaction between RA and RO could involve direct cell-cell contact or a soluble factor. To date, two pieces of evidence have indicated an interaction between CD45R subsets. Firstly, Wallace and Beverley demonstrated that RA^+ T cells could be induced to express CD54 and CD25 in response to CD2 MAbs providing activated RO^+ T cells were present (Wallace and Beverley, 1990). In the absence of anti CD2 or activated RO^+ T cells, increases in CD54 or CD25 expression on RA^+ T cells were not observed. This data suggested that RO^+ cells could recruit RA^+ cells for activation. Secondly, activation by antigens including alloantigen leads to the release of IL-2 by RA^+ T cells and IL-4 by RO^+ T cells (Akbar et al. 1991; Swain et al. 1991; Constant et al. 1994). The addition of IL-2 to alloresponsive RO^+ T cells enhances proliferation but has little effect on RA^+ alloresponses. IL-4 had the opposite effect. In addition, the reconstitution of CD45R subsets produced proliferation that was greater than the cumulative values of RA and RO proliferation separately (Akbar et al. 1991).

From the above evidence it was concluded that CD45R subset communication is based on cytokine release and results in enhanced proliferation. The following experiments attempt to investigate the interaction between CD45R subsets in more detail.

EXPERIMENTAL DESIGN

Cultures were set-up as described in Chapter 2. The cell numbers used in each type of mixing experiment are shown in Table 4-I.

The following calculation was used to determine co-operation /interaction between the CD45R subsets.

$$\text{Interaction} = \frac{\text{cpm (D-E)}}{[\text{cpm (B-F)}] + [\text{cpm (C-G)}]}$$

A value greater than 1 indicates a positive interaction. The letters in the equation indicate cell populations described below in Table 4-I.

Table 4-I: The number of responder T cells in the interactive experiments.

Experiment	Stimulators	$CD45RA^+$	$CD45RO^+$	PBMC
A	10^5	-	-	10^5
B	10^5	10^5	-	-
C	10^5	-	10^5	-
D	10^5	$5x10^4$	$5x10^4$	-
E	-	$5x10^4$	$5x10^4$	-
F	-	10^5	-	-
G	-	-	10^5	-

Values represent the number of each cell type per well in a flat-bottomed 96 well plate. Each experiment was carried out in triplicate.

RESULTS

Equal numbers of $CD4^+$ RA^+ and RO^+ T cell subsets were recombined in a primary MLR and stimulated with either $DR1^+$ LCL, $DR1^+CD54^+$ TFCL or $DR1^+$ TFCL. These responses were compared to proliferation by $1x10^5$ whole PBMC, $CD4^+RA^+$ or $CD4^+RO^+$ T cells against the same stimulators (Table 4-II). The reconstitution of equal proportions of CD45R subsets produced responses of similar magnitude to whole PBMC against all three stimulator cell types.

The reconstitution of CD45R subsets against $DR1^+$ LCL and $DR1^+CD54^+$TFCL gave an interaction value below 1 for all four donors. The interaction values were greater than one, when $DR1^+$TFCL was the stimulus;in three experiments the interaction value was between 1.05 and 1.4. The fourth donor had a much higher value of 3.1. Clearly, some interaction occurs when RA^+ and RO^+ cells are stimulated with $DR1^+$ TFCL but not with $DR1^+$ $CD54^+$ TFCL or $DR1^+$ LCL.

To determine what ratio of each CD45R subsets was required to induce maximum interaction, increasing numbers of RA^+ T cells were mixed with decreasing numbers of RO^+ T cells in a primary MLR (Fig. 4-1A & B). The mean proliferation value peaked when $6x10^4$ RA^+ cells were combined with $4x10^4$ RO^+ T cells against all three stimuli tested ($DR1^+$ LCL, $DR1^+CD54^+$ TFCL and $DR1^+$ TFCL). The mixture of RA^+ and RO^+ T cells in a 3:2 ratio was significantly greater than either of the $CD4^+CD45R$ subsets alone but not significantly different from responses of $1x10^5$ whole PBMC.

To investigate this interaction further, MMC was used to treat either CD45R subset in further recombination studies. Untreated RA^+ cells plus MMC treated RO^+ cells stimulated with $DR1^+$ LCL (Fig. 4-2A), resulted in a reduction in proliferation with decreasing numbers of untreated cells. Similar patterns occurred for untreated RO^+ populations mixed with MMC treated RA^+ cells. There was a progressive fall in the response as untreated cells were diluted with greater numbers of MMC treated cells. When the above experiment was repeated using $DR1^+$ TFCL, the result was similar (Fig. 4-2B). The experiments indicate that synthetic and proliferative capabilities were essentail for an interaction.

Table 4-II: Interaction between CD45RA and CD45RO T cells in an MLR, expressed as mean ± SEM of cpm [^{3}H] TdR.

Responder 1	**DR1$^+$LCL**	**DR1$^+$CD54$^+$TFCL**	**DR1$^+$TFCL**	**Control**
A. PBMC	14123 ± 210	9634 ± 358	8108 ± 352	1085 ± 126
B. RA	11797 ± 285	4112 ± 116	1185 ± 270	123 ± 13
C. RO	12952 ± 370	4881 ± 260	1209 ± 196	128 ± 15
D. RA + RO	13779 ± 482	8569 ± 274	7611 ± 210	737 ± 15
Interaction *	0.53	0.9	3.21	

Responder 2	**DR1$^+$LCL**	**DR1$^+$CD54$^+$TFCL**	**DR1$^+$TFCL**	**Control**
A. PBMC	32643 ± 1271	5732 ± 149	3039 ± 244	100 ± 17
B. RA	29001 ± 247	3747 ± 310	1435 ± 140	242 ± 12
C. RO	31409 ± 777	3135 ± 473	1231 ± 34	178 ± 82
D. RA & RO	34664 ± 1050	5807 ± 45	3063 ± 387	699 ± 73
Interaction *	0.56	0.79	1.05	

Responder 3	**DR1$^+$LCL**	**DR1$^+$CD54$^+$TFÇL**	**DR1$^+$TFCL**	**Control**
A. PBMC	22938 ± 1235	7197 ± 255	3995 ± 125	128 ± 23
B. RA	20113 ± 2251	4911 ± 136	1881 ± 254	397 ± 58
C. RO	20399 ± 1955	4715 ± 188	1495 ± 272	284 ± 100
D. RA & RO	23014 ± 1657	6928 ± 314	3736 ± 218	664 ± 97
Interaction *	0.57	0.77	1.39	

Responder 4	**DR1$^+$LCL**	**DR1$^+$CD54$^+$TFCL**	**DR1$^+$TFCL**	**Control**
A. PBMC	36421 ± 1077	8498 ± 308	5384 ± 266	222 ± 98
B. RA	34999 ± 985	5842 ± 424	2580 ± 254	369 ± 81
C. RO	33218 ± 1456	5799 ± 269	2433 ± 284	321 ± 101
D. RA & RO	35842 ± 1288	8107 ± 411	5497 ± 211	503 ± 83
Interaction *	0.53	0.74	1.1	

* For interaction formula see Section 4-2.

DISCUSSION

RA^+ and RO^+ T cells are known to respond with similar intensity to alloantigen (Akbar et al 1989; Sanders et al 1988; Merkenschlager et al 1988) but this is usually at a lower level of proliferation than whole PBMC. The combination of equal numbers of CD45R subsets significantly increased responses in the majority of cases and gave similar proliferation levels to that of whole PBMC and significantly greater than either CD45R subset alone. At first glance, reconstitution of CD45R subsets indicates an interaction between RA^+ and RO^+ T cells against alloantigen. Assessment of the co-operative effect using the formula described previously, shows that equal numbers of CD45R subsets stimulated with DR^+ LCL produces a value close to one. This is an additive effect and shows that responses from the recombination of RA^+ and RO^+ T cells is no greater than the sum of individual CD45R populations. When the same calculation is used on responses to $DR1^+$ TFCL, the value was greater showing that the effect was a net positive interaction. Determination of the ratio of CD45R subsets required for maximum proliferation found that increasing numbers of RA^+ T cells with decreasing numbers of RO^+ cells, produced a peak response when RA^+ cells were in a slight majority. The response curves from activation with either stimuli indicate that approximately equal numbers of CD45R subsets are sufficient for enhanced stimulation with a bias towards the RA^+ population.

MMC treatment of either CD45R subsets inhibits the interaction between the populations indicating a requirement for DNA synthesis prior to or during the co-operative effect. This suggests that either that a.) the interaction involves DNA synthesis of both subsets or b.) a soluble factor produced by one subset enhances the proliferation of the other. MMC treatment not only prevents cell division but the production of all new proteins (Tomasz et al. 1987; Veda and Komano, 1984). If the interaction involved cell-cell contact, molecules involved in this interaction are either not expressed on resting cells, or the density of expression is too low to be detected. Since reconstituted subsets stimulated with $DR1^+$ TFCL show the greatest positive interaction, it is reasonable to assume that an interaction between RA^+ and RO^+ T cells is most effective when responder cells are provided with a sub-optimal stimulus.

An alternative mechanism for the co-operative effect involves a soluble factor released by one or both of the CD45R populations. Studies by Akbar *et al* demonstrated that RO^+ proliferation is up-regulated by rIL-2 (Akbar et al. 1991). Since IL-2 is the principal cytokine released by RA^+ T cells and RO^+ produce mainly IL-4 (Sanders et al. 1988; Budd et al. 1987; Yssel et al. 1992; Luqman and Bottomly, 1992), it is likely that combinations of CD45R subsets would release the necessary cytokines to cause complementary enhancement of the opposite subsets response. Therefore if a cytokine was the mediator between CD45R populations, a co-operative effect would be prevented by MMC treatment. Since both populations behaved in a similar manner when treated with MMC, it is clear that the interaction is mutually beneficial and not a one-sided interaction.

The present study shows that CD45R subsets can interact and provide accessory signals for one another. The exact identity of this signal is unknown.

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Fig. 4-1

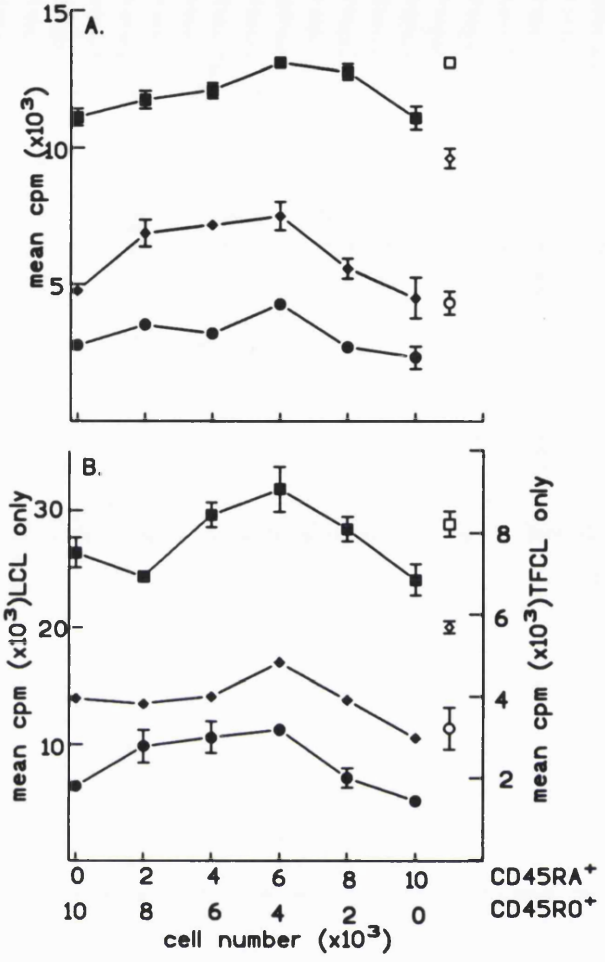

Figure 4-1A & B: Interaction between CD45R subsets.

Mixtures of increasing numbers of $CD4^+RA^+$ T cells and decreasing numbers of $CD4^+RO^+$ T cells from two separate HLA-DR1 negative donors (4-1 A & B) against $DR1^+$ LCL (■-■); $DR1^+CD54^+$ TFCL (◆-◆) or $DR1^+$ TFCL (●-●). Open symbols represent the same stimuli with $1x10^5$ whole PBMC as the responder population.

Fig. 4-2

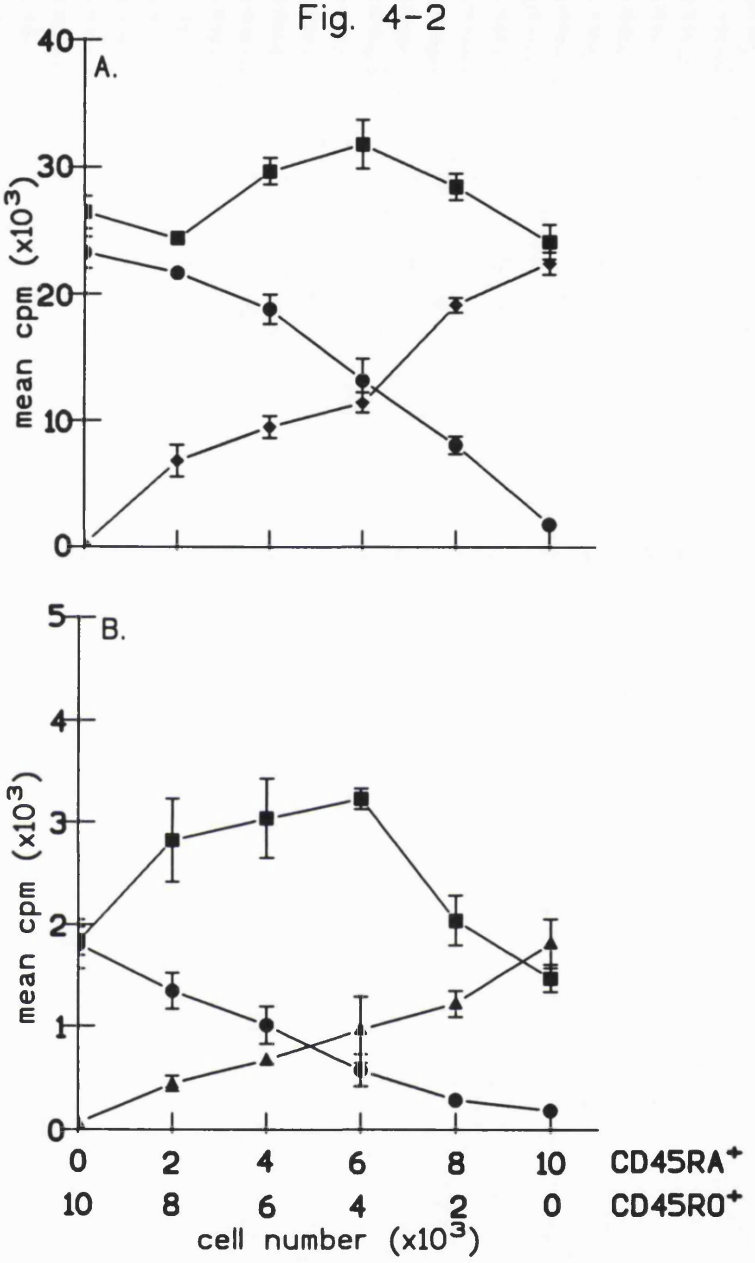

Figure 4-2A& B: The effect of MMC treatment on CD45R subset interaction.

Variable proportions of $CD4^+RA^+$ T cells $CD4^+RO^+$ T cells in the absence of MMC treatment (■-■); or with $CD4^+$ RA^+ MMC treated cells (●-●) or $CD4^+$ RO^+ MMC treated T cells (◆-◆) together with untreated cells of the opposite subset stimulated with A.) $DR1^+$ LCL or B.) $DR1^+$ TFCL.

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CHAPTER 5

THE EFFECT OF CYTOKINES ON T CELL RESPONSES TO TFCL.

INTRODUCTION

T cell activation induces naive T cells to synthesise IL-2 which behaves in an autocrine fashion, enhancing the expression of the IL-2 receptor (IL-2R) and inducing clonal expansion and differentiation into effector cells (Minami et al. 1993; Waldmann, 1991; Ehlers and Smith, 1991). Differentiation of $CD4^+$ T cells into distinct $CD4^+$ T_H cell populations follows. These populations can be distinguished on the basis of cytokine profile and function. T_H1 cells are involved in cell mediated immunity and predominantly release large amounts of IL-2 and smaller concentrations of IFNγ. T_H2 cells mainly produce IL-4, the main function of T_H2 cells is the induction of antibody formation in B cells (Paliard et al. 1988; Umetsu et al. 1988; Bacchetta et al. 1990). The T_H subsets were first described in the mouse by Mosmann (Mosmann et al. 1986). Subsequent studies have shown that T_H2 cells also produce IL-5, IL-6 & IL-10 in addition to IL-4. Some cytokines such as IL-3 and GM-CSF are produced by both populations (Cherwinski et al. 1987). The division of $CD4^+$ T cells by cytokine profile in humans is ill defined and largely dependent upon the stimulant. The strongest evidence for this separation is in allergy and parasitic disease where T_H2 like cells produce IL-4 (Salgame et al. 1991).

Naive T cells take up to days to develop into effector cells. During this period, cells not only switch from RA^+ to RO^+, but also alter in cytokine profile. Whereas naive RA^+ T cells predominantly produce IL-2, RO^+ T cells synthesise IL-2, IL-4, IL-6 and IFNγ, although IL-4 and IFNγ are the principal products (Akbar et al. 1991; Ehlers and Smith, 1991). In humans, the switch from RA^+ to RO^+ is also accompanied by a decrease in the expression of RB. As effector T cells undergo clonal expansion the expression of this molecule declines. Once again the cytokine profile of these cells alters. The differentiation from $RA^+RB^+RO^-$ to $RA^-RB^+RO^+$ is accompanied by a decrease in IL-2 synthesis, an increase of IL-4 and detectable levels of IFNγ. A further switch to so-called late primed T cells with a RA^- $RB^{lo}RO^+$ phenotype ceases IL-2 production, lowers IL-4 production and increases IFNγ (Salmon et al. 1994).

Early primed cells are thought to be T_H2-like cells and late primed cells that release high levels of IFNγ are thought to be T_H1-like cells (Paul and Seder, 1994). RO^+ T cells produce maximum levels of IL-2 within 24hrs of stimulation. RA^+ T cells release the majority of IL-2 on day 2 (Swain et al. 1991; Akbar et al. 1991). In GvHD, donor CD4 T cells release IL-2 when exposed to alloantigen and gradually switch to a T_H2-like cytokine profile. Both acute and chronic GvHD appear to begin with a T_H2-like profile (Rus et al 1995).

The IL-2 consists of three membrane components, the α, β and γ chains. Resting T cells do not express the α chain of the IL-2R. Upon activation, the α chain is induced and the heterodimer is expressed, allowing T cells to respond to much lower levels of IL-2 (Minami et al. 1993). Both CD45R subsets express equivalent levels of IL-2R although as with IL-2 production, RA^+ T cells peak later than RO^+ T cells (Akbar et al. 1991; Akbar et al. 1989).

A number of cytokines are known to enhance T cell activation and proliferation (see Table 5-I). IL-4, IL-6, IFNγ and TNFα can costimulate T cells in the presence of cross-linked CD3 MAbs, CD2 MAbs or sub-optimal doses of mitogens (Rodriguez et al. 1990; Tosato and Pike, 1988; Uyttenhove et al. 1988; Dao et al. 1993; Lee et al. 1986; Scheurich et al. 1987). Moreover, IL-6 induces T cells to be more responsive to low levels of IL-2 (Roth, 1994). In combination with IL-4, IFNγ and PHA, IL-6 can cause dramatic increases in proliferation. Low levels of proliferation against CD2 MAbs can also be supported by IL-6.

IL-1 exhibits co-stimulatory effects on T cells in the presence of autologous APC or sub-optimal doses of mitogens (Tosato et al. 1990), inducing IL-2 production and proliferation (Kaye and Janeway, Jr. 1984; Kurt-Jones et al. 1987; Lichtman et al. 1988). IL-1 is a major factor in primary responses to soluble antigens such as KLH. The effect is inhibited by MAbs against IL-1β (Plebanski et al. 1992; Dinarello, 1991). Initially IL-1 was believed to play an essential role in T cell activation by APCs (Tosato et al. 1990) however, the most potent APC's (dendritic cells) do not release IL-1 and still effectively activate T cells (Mizel, 1987). IL-1 alone cannot replace monocytes when T cells are stimulated with CD3 MAbs. It is therefore likely that monocyte derived IL-1 can enhance proliferation and the production of IL-2 but is not an essential component (Endler-Jobst et al. 1991).

The evidence presented in the previous chapter, showed an interaction between RA^+ and RO^+ populations. Experiments in this Chapter were designed to investigate if any one cytokine can replace the effect of the reciprocal CD45R subset in response to minimal stimulation. Human recombinant cytokines were tested in the transfectant model to investigate if this effect is based on cytokine production and to examine any potential differences in cytokine requirements by CD45R subsets.

EXPERIMENTAL DESIGN

Alloantigen and PHA stimulated PBMC and CD45R subsets were stimulated in primary MLRs in the presence or absence of either IL-1β, IL-2, IL-4, IL-6 or IFNγ. Proliferation of PHA stimulated cells was assessed on day 3 and alloantigen stimulated cells on day 5, as previously described (see Section 2-5). Data is represented as the mean cpm of [^{3}H]TdR uptake. The IL-2 dependent cell line CTLL was obtained from the ECACC.

Fig. 5-1

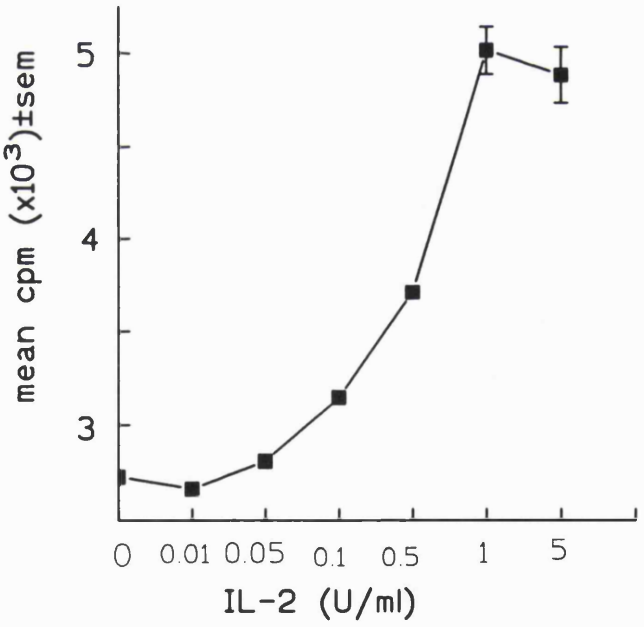


Figure 5-1: Dose response of IL-2 stimulation of CTLL cells.
Titration of recombinant IL-2 by the IL-2 dependent cell line CTLL. Proliferation was measured by [^{3}H]TdR uptake of triplicate wells. Symbols represent mean ± SEM proliferation.

Table 5-I: Cytokines involved in T cell activation.

Cytokine		Structure and origin	Effect on T cells	Reference
IL-1β	Origin	Monocytes	↑ IL-2R expression and IL-2 production;	(Hagiwara et al. 1987; Tosato et al. 1990;
	Size	17.5 kDa.	↑ IL-3 gene expression;	Luqman et al. 1992; Mizel, 1987;
	Receptor	Expressed on T cells, B cells & monocytes.	Co-stimulation for CD2 and CD3 MAbs	Dinarello, 1989)
IL-2	Origin	T cells and NK cells	Induces proliferation;	(Minami et al. 1993; Robb and Smith,
	Size	15-17 kDa.	Enhances motility;	1981; O'Flynn et al. 1985; Paul and Seder,
	Receptor	Expressed on T cells, monocytes and granular lymphocytes.	Stimulation of CTL activity;	1994; Platts et al. 1993; Roth, 1994; Akbar
			↑ cytokine production (IL-2, IL-4 & IFNγ);	et al. 1991; Smith, 1992; Taniguchi and
			Promotion from G_1 to S phase.	Minami, 1993)
IL-4	Origin	T cells and thymocytes	Maintenance and proliferation of activated T cells.	(Ben-Sasson et al. 1990; Bazan, 1990; Paul
	Size	15-19 kDa	Inhibition of IFNγ, TNFα, TNFβ and GM-CSF prod'n;	and Seder, 1994; Akbar et al. 1991)
	Receptor	Expression on T cells, thymocytes, NK, B and monocytes.	Enhances CTL activity;	
			Co-stimulation activity with CD3 MAb and PHA stimulation.	
IL-6	Origin	T and B cells, monocytes, fibroblasts and endothelial cells.	Proliferation;	(Tosato and Pike, 1988; Tosato et al. 1988;
			Co-stimulation with PHA or Con A;	Uyttenhove et al. 1988; Gauldie et al. 1987;
	Size	22-29 kDa	Induces IL-2 production and IL-2R expression;	Lorre et al. 1990; Wong and Clark, 1988;
	Receptor	Expressed on T, NK, monocytes	Enhancement of CTL production.	Van Damme et al. 1987; Mosmann et al. 1986; Sideras et al. 1988; Seder et al. 1992)
IFNγ	Origin	T and NK cells	Enhanced expression of MHC class I and class II;	(Ijzermans and Marquet, 1989; Gray et al.
	Size	20-25 kDa	Up-regulation of IL-2R on PHA stimulated cells;	1989; Adams and Hamilton, 1987;
	Receptor	Expressed on most leukocytes	Inhibition of IL-4 and IL-5 production;	Rodriguez et al. 1990)
			Proliferation; Enhancement of CTL production.	

* 'Receptor' indicates the cell types expressing receptors for that particular cytokine.

The effect of each cytokine on allogeneic T cell activation was assessed using the following formula:

$$\text{Cytokine stimulation ratio} = \frac{[^3H]\ TdR\ test + cytokine}{[^3H]\ TdR\ control}$$

Control = test response in the absence of recombinant cytokines.

RESULTS.

Stimulation of CTLL in a 3 day proliferation assay gave optimal proliferation with one U/ml IL-2 (Fig. 5-1). The remaining cytokines were titrated on PHA (Fig. 5-2) or alloantigen (Fig. 5-3) stimulated PBMCs. Optimal concentrations of cytokines under these conditions were as follows; IFNγ (50 U/ml), IL-1 (5 U/ml), IL-4 (0.5 to 1 μg/ml) and IL-6 (5 U/ml) for either PHA or alloantigen stimulation.

Five HLA-DR1 negative donors were stimulated in primary MLRs with TFCL or $DR1^+$ LCL in the presence or absence of each of the above cytokines (Fig. 5-4). The addition of either IL-1β or IL-6 had no effect on responses by PBMC or CD45R subsets to alloantigen (Fig. 5-4A and 5-4D). IL-2 however, enhanced the responses of CD45R populations against all TFCL (Fig. 5-4B). This effect was less apparent in whole PBMC responses. The addition of IL-2 to $CD4^+$ CD45R subsets had the greatest effect when the stimulator populations were either FCL or $DR1^+$ $CD58^+$ TFCL. IL-2 did not have such a marked effect on PBMC or $CD4^+$ CD45R subset responses against $DR1^+$ TFCL or $DR1^+$ LCL.

Responses by RA^+ T cells in the presence of IL-4 were enhanced between 2 and 3 fold (Fig 5-4C). PBMC proliferation was not significantly affected by the presence of IL-4 under any of the conditions tested. RO^+ responses were increased by the addition of IL-4 although the cytokine ratios were between 1.5 and 2 fold lower than RA^+ responses. Of all the stimulator populations, $DR1^+$ TFCL profited the most from the addition of IL-4 for PBMC, $CD4^+RA^+$ and $CD4^+RO^+$ T cells.

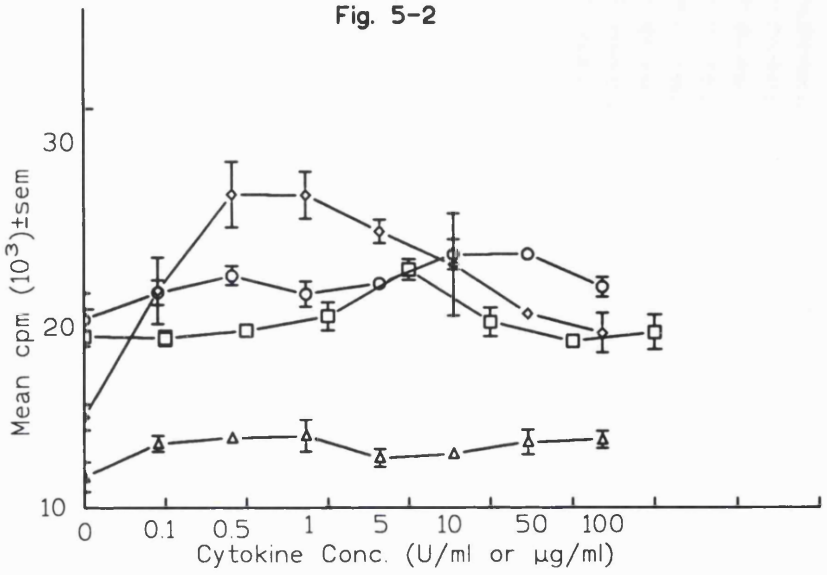


Figure 5-2: Dose response of recombinant cytokines on PHA stimulated PBMC.

The titration of IFNγ (square), IL-1β (triangle), IL-4 (diamond) and IL-6 (circle) on PHA stimulated PBMC. Each point represents[^{3}H]TdR uptake shown as mean cpm ± SEM for triplicate wells.

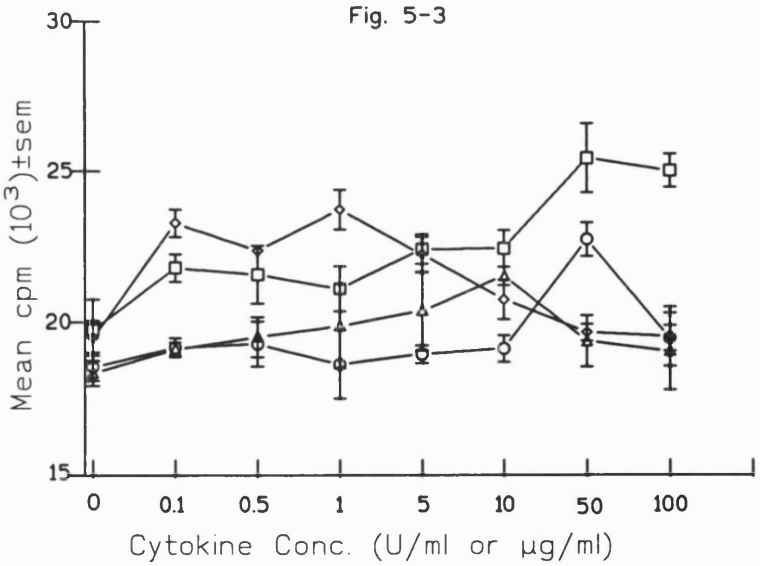


Figure 5-3: Titration of recombinant cytokines by alloantigen stimulated PBMC.

Symbols- see Fig. 5-2.

IFNγ had the greatest effect on RO^+ T cells when stimulated with alloantigen. Responses by RO^+ however, against the control cell line FCL, were not enhanced by the presence of IFNγ (Fig 5-4E). RO^+ T cell responses against $DR1^+CD58^+$ TFCL exhibited the greatest effect.

In general, PBMC responses did not demonstrate such dramatic enhancement in the presence of recombinant cytokines. Also, the presence of any of the recombinant cytokines to $DR1^+$ LCL stimulated PBMC, RA^+ and RO^+ T cells had little effect. Collective analysis of the effects of cytokines on T cell subset responses to TFCL or $DR1^+$ LCL however, did not demonstrate any statistically significant conclusions. This was due to wide variations seen between different individuals. Stimulation of PBMC and CD45R T cell subsets by $DR1^+CD54^+$ TFCL and $DR1^+CD54^+$ TFCL produced similar levels of proliferation (data not shown for $DR1^+CD54^+$ TFCL).

The number of individual donors that showed a positive augmentation to each cytokine were also analysed (Table 5-II). Results were variable, although proliferation in the presence of IL-1β or IL-6 were consistently poor with all responder populations. IL-2 enhanced responses against all the stimulator populations, $DR1^+$TFCL, $DR1^+CD58^+$ TFCL and $DR1^+$ LCL in particular. IL-4 augmented RA^+ responses reflecting conclusions drawn from the collective analysis, although responses to $DR1^+CD58^+$TFCL by T cell subsets in the presence of IL-4 were poor with only one donor responding as whole PBMC or RO^+. Of all the cytokines examined, IFNγ induced the most significant effect, with all five donors showing a net positive effect when stimulated with $DR1^+$LCL ($CD4^+$CD45R subsets only). A distinction between RA^+ and RO^+ T cells was also observed in the number of donors responding to IL-4 and IFNγ.

DISCUSSION.

The addition of recombinant cytokines had variable effects on alloactivation, these effects were largely dependent upon the type of responder cells. Overall, PBMCs did not benefit as greatly as CD45R populations to the presence of cytokines.

Table 5-II: The number of donors demonstrating a positive proliferative response to recombinant cytokines.

A. FCL

Cytokine	PBMC	$CD45RA^+$	$CD45RO^+$
IL-1β	1	0	0
IL-2	2	3	1
IL-4	1	3	1
IL-6	1	0	0
IFNγ	3	4	2

B. $DR1^+$TFCL

Cytokine	PBMC	$CD45RA^+$	$CD45RO^+$
IL-1β	0	0	0
IL-2	3	4	3
IL-4	1	2	2
IL-6	1	0	1
IFNγ	1	4	4

C. $DR1^+CD58^+$ TFCL

Cytokine	PBMC	$CD45RA^+$	$CD45RO^+$
IL-1β	1	0	1
IL-2	4	2	2
IL-4	1	0	1
IL-6	2	0	2
IFNγ	4	2	3

D. $DR1^+$ LCL

Cytokine	PBMC	$CD45RA^+$	$CD45RO^+$
IL-1β	1	0	1
IL-2	5	3	5
IL-4	3	5	3
IL-6	1	1	2
IFNγ	3	5	5

Numerals represents the number of donors out of a total of five, that showed a statistically significant increase in proliferation by the addition of cytokines using a nonparmetric t-test. Calculations were based on the cytokine ratios in Fig. 5-4. where a cytokine ratio greater than 2.5 was considered a postive effect.

Fig. 5-4

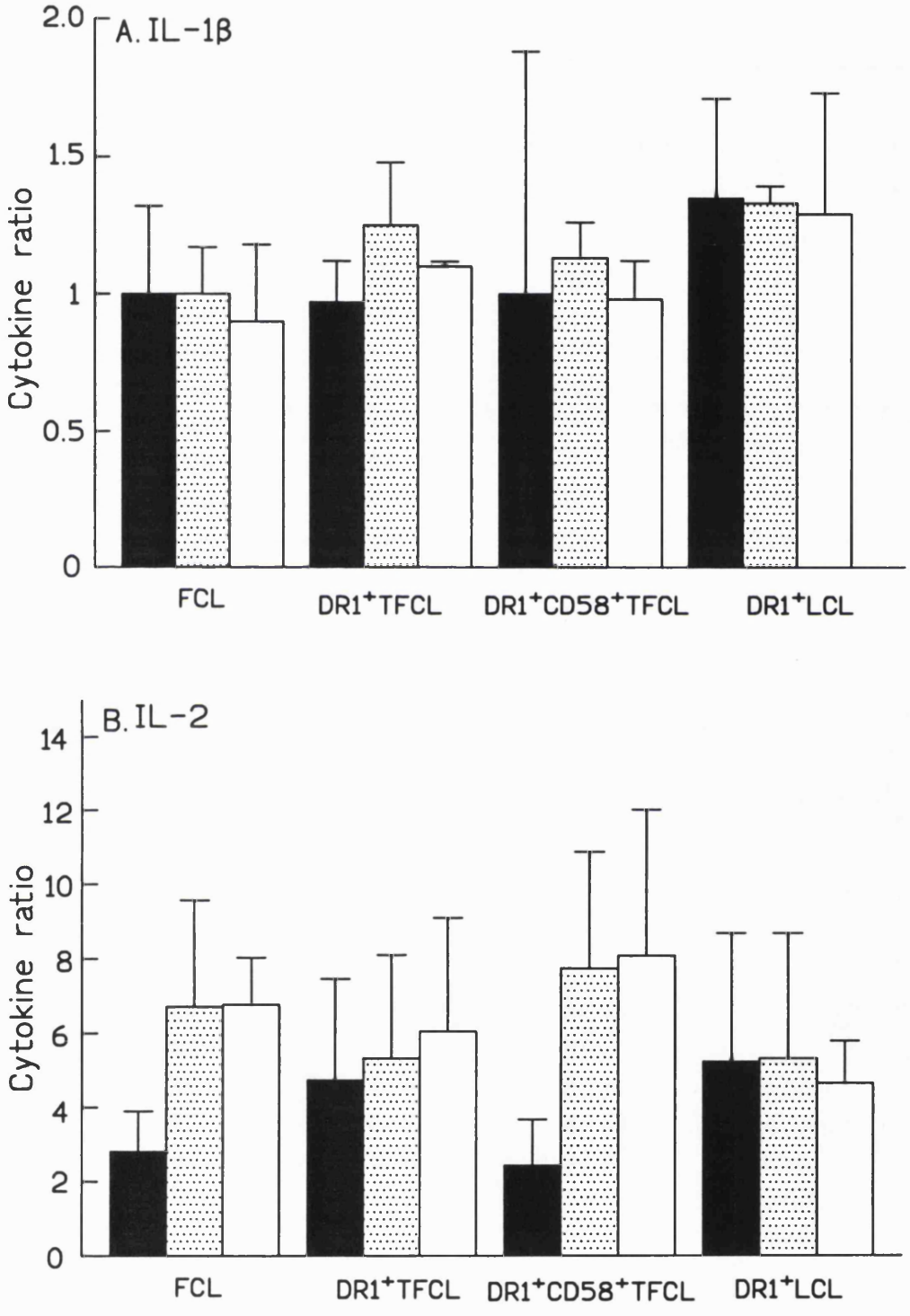
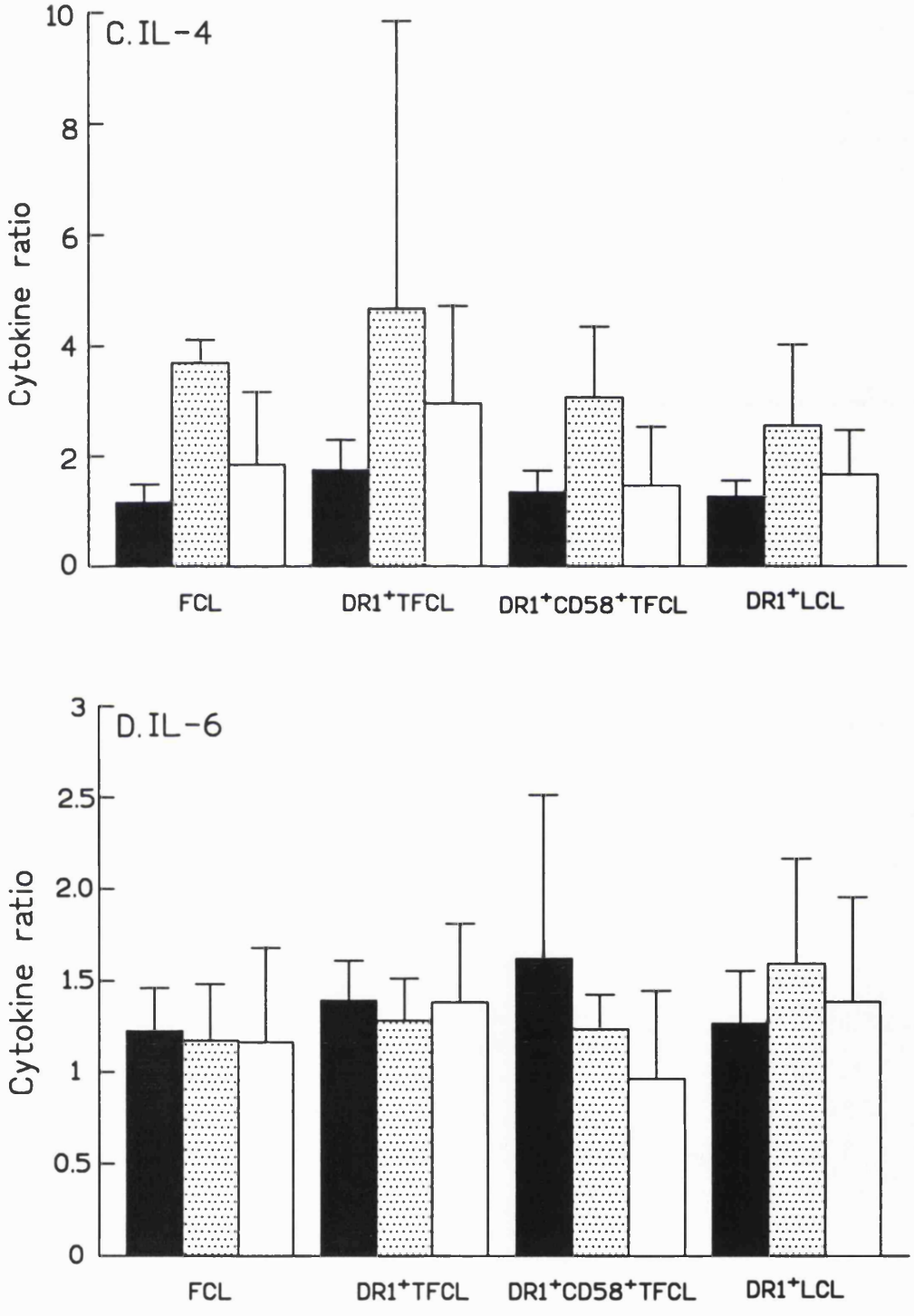

A. IL-1β
Cytokine ratio
2.0
1.5
1
0.5
0
FCL
DR1+TFCL
DR1+CD58+TFCL
DR1+LCL
B. IL-2
Cytokine ratio
14
12
10
8
6
4
2
0
FCL
DR1+TFCL
DR1+CD58+TFCL
DR1+LCL

Fig. 5-4 cont'd


C. IL-4
Cytokine ratio
10
8
6
4
2
0
FCL
DR1+TFCL
DR1+CD58+TFCL
DR1+LCL
D. IL-6
Cytokine ratio
3
2.5
2.0
1.5
1
0.5
0
FCL
DR1+TFCL
DR1+CD58+TFCL
DR1+LCL

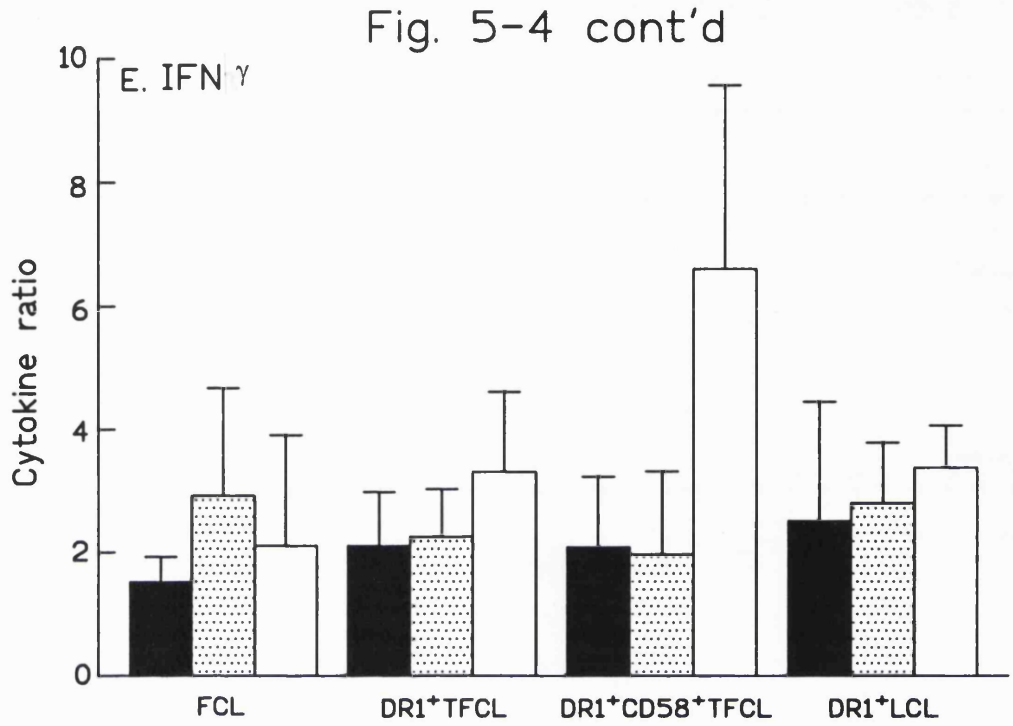


Figure 5-4: The effect of recombinant cytokines on alloantigen responses.
The effect of A.) IL-1β , B.) IL-2, C.) IL-4, D.) IL-6 and E.) IFNγ on PBMC (solid bars), RA^+ (speckled bars) and RO^+ (open bars) T cell populations stimulated with TFCL or $DR1^+$ LCL. Each bar represents the cytokine ratio calculated as described in Section 5-2 from five HLA-DR1 negative donors. ** $p<0.01$; * $p<0.05$ using Kruskal-Wallis non-parametric AOV.

It is likely that whole PBMCs provide the optimal activation signals including cytokine release to induce maximum proliferation against alloantigen. Separation of T cells into CD45R populations demonstrated a dependence for IL-2 by both CD45R T cell populations. Responses against $DR1^+CD58^+$ TFCL and FCL were augmented most by the presence of IL-2 although all stimulator populations tested demonstrated a net positive effect. It is well documented that IL-2 is a vital component of T cell activation (Minami et al. 1993; Waldmann, 1991). Both CD45R subsets proliferate vigorously to alloantigen in the presence of IL-2. Conversion of RA^+ to RO^+ has been observed with RA^+ T cell cultures whilst RO^+ T cells maintain RO expression. The addition of either IL-4 or IL-6 to purified CD45R populations does not affect CD45R expression or induce proliferation (Nguyen et al. 1993). IL-2 also augments CD3 stimulated RA^+ T cell cultures (Wasik and Morimoto, 1990). In conjunction with my own studies, the above reports demonstrate the potent abilities of IL-2 with respect to CD45R T cell proliferation. However, stimulation with IL-2 and alloantigen cannot differentiate between CD45R populations.

The presence of either IL-1β or IL-6 had no significant effect on T cell proliferation against alloantigen by any of the responder populations. Previous studies have noted that in combination, IL-1 and IL-6 can enhance CD2 mediated pathways of activation (Endler-Jobst et al. 1991). It was thought that the addition of either of these cytokines would amplify the effect of the double transfectants $DR1^+CD58^+$ TFCL or $DR1^+CD54^+$ TFCL, but this was not the case. The combination of IL-1 and IL-6 has been previously shown to have an additive effect upon mitogen stimulated cells when present in low concentrations (Endler-Jobst et al. 1991). IL-4 and IL-6 also augment proliferation of RA^+ cells to IL-2 in the presence of CD3 MAbs (Wasik and Morimoto, 1990).

It is possible that combinations of cytokines during T cell activation may be involved in determining the fate of a particular T cell (see Fig. 1-6). However, the effects of the combination of various cytokines was not examined here.

CD45R subset stimulation by alloantigen was distinguished on the basis of IL-4 and IFNγ effects. RA^+ T cells showed a clear, although not statistically significant, augmentation in responses to alloantigen in the presence of IL-4 that was not seen with RO^+ T cells. In contrast, RO^+ T cells responded strongly to IFNγ whereas RA^+ responses were comparable to those of PBMCs. Distinctions between RA^+ and RO^+ T cells with respect to IL-4 responses have also been reported in CD3 and CD2 MAb stimulated cultures. Similar to the results reported here, RA^+ cells responded strongly in the presence of IL-4. RO^+ T cells were not affected by the addition of IL-4. Such differences between CD45R subsets may be due to variation in cytokine receptor expression. IL-2R expression is upregulated on both CD45R subsets following activation, IL-4 or IFNγ receptor expression may also vary with CD45R isoform expression. The expression of IL-4R and IFNγR on CD45R T cell subsets has not been investigated here. Differentiation between human CD45R T cell subsets has been previously identified with respect to cytokine release. $CD4^+RA^+$ T cells primarily release IL-2 whilst RO^+ T cells produce either IL-4 or IFNγ, depending upon the stage of priming. Early primed T cells expressing RO release large amounts of IL-4.

IL-2, IL-4 and IFNγ all enhanced responses to FCL by PBMC and CD45R subsets. IL-4 was the only cytokine to show a clear effect on $DR1^+$ TFCL stimulated cells and this only occurred with CD45R populations. The addition of other cytokines did not appear to compensate for the lack of accessory molecules expressed on the APC. Individual donors responded strongly to IFNγ as CD45R subsets against $DR1^+$ $CD58^+$ TFCL, four out of five donors demonstrated significant increases in proliferation due to the addition of IFNγ.

The addition of a co-stimulus in the form of CD54, coupled with recombinant cytokines also had a limited effect, although on an individual basis, IL-2 and IFNγ showed the increase in proliferation.

$DR1^+$ LCL responses were employed as a means of determining T cell responses with the optimal MHC presentation with and without accessory signals. in these circumstances, the majority of responses were enhanced by the addition of IL-2, IL-4 or IFNγ. However, inter-individual variation was great and enhancements by cytokines were obscured when all five donors were collectively assessed. This makes it difficult to draw clear cut conclusions from the $DR1^+$ LCL data. On one hand it is not surprising that collective examination revealed that the presence of cytokines have little effect when optimal signals are provided by the $DR1^+$ LCL. Alternatively, individuals showed that IL-2, IL-4 and IFNγ can enhance responses against $DR1^+$ LCL showing that T cell responses to such stimuli in the absence of cytokines are not maximal.

Despite increasing trends in T cell responses to TFCL in the presence of IL-2, IL-4 and IFNγ, none of the enhancements were statistically significant. Perhaps with an increased sample number a significant effect would be seen. Analysis of responses on an individual basis showed that cytokines increased responses to alloantigen by varying degrees. This phenomenon was also observed in the preliminary experiments using T cells subsets against alloantigen alone (see Chapter 3). Natural variation between individuals tends to overshadow the effects of TFCL and cytokines against T cell subsets when data is analysed collectively.

Further studies addressing questions such as how the combination of cytokines affect responses and cytokine receptor expression on CD45R T cell populations may advance the understanding of the distinction between CD45R subsets identified in this study.

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CHAPTER 6.

THE EFFECT OF AUTOLOGOUS APC AND CD28 ON ALLOANTIGEN ACTIVATION.

INTRODUCTION.

T cells recognise and respond to antigenic peptide in the MHC groove of antigen presenting cells (APC). The APC also provides the T cell with secondary signals such as CD28-CD80, CD2-CD58 pathways or cytokines such as IL-1 (Kampschmidt, 1984; Bockenstedt et al. 1988; Linsley et al. 1992; Galvin et al. 1992; Moingeon et al. 1989; Mueller et al. 1989). There are a number of cell types able to act as APCs, most of the literature available describes monocytes, dendritic cells (DCs) and B lymphocytes as the most effective (Inaba and Steinman, 1984; Metlay et al. 1989; Finkelman et al. 1992; Liu and Janeway, Jr. 1991; Huges and Pober, 1993). These cells express high levels of MHC class I & II in addition to a wide range of adhesion and accessory molecules. In humans, dendritic cells are thought to be the most potent APC for alloantigen and soluble antigen specific T cells. However, peripheral blood DCs and monocytes are comparable in their ability to stimulate T cells with alloantigen or anti-CD3 MAbs (Thomas et al. 1993; Van Voorhis et al. 1982; Guidos et al. 1984).

Alloantigen stimulation can proceed by one of two pathways; direct or indirect (see Chapter 1.12). Direct allorecognition involves the alloantigen being presented by foreign APC where the T cell recognises the MHC and any bound peptide as foreign. Indirect antigen presentation involves the processing and presentation of foreign MHC by host APC (Marrack and Kappler, 1988; Lechler et al. 1990; Sayegh et al. 1994). Differences between $CD4^+$ and $CD8^+$ T cells have been identified with respect to their ability to recognise antigen by the direct or indirect route (Via et al. 1990). $CD4^+$ T cells recognise alloantigen by either route whereas $CD8^+$ T cells can only recognise and respond to alloantigen on foreign APC. It is possible that RA^+ and RO^+ cells may also differ.

RA^+ and RO^+ $CD4^+$ T cells require different types of antigen presenting cells in certain situations. RA^+ T cells are more dependent on 'professional APC' such as dendritic cells.

RO^+ cells can utilise cells where expression of MHC is induced through activation in addition to professional APCs (Damle et al. 1992; Ronchese and Hausmann, 1993; Larsen et al. 1992). Such cells include activated T, B cells and endothelial cells.

Low levels of proliferation against the $DR1^+$ TFCL, $DR1^+$ $CD58^+$ and $DR1^+$ $CD54^+$ TFCL cells by CD45R populations may be due to their inability to respond to alloantigen presented in this manner in the absence of self APC, since purification of CD45R populations removes the majority of APCs. The effect of autologous APC on alloantigen responses is discussed below.

Accessory signals or co-stimuli are vital to T cell activation (Jenkins and Johnson, 1993; Linsley and Ledbetter, 1993). Removal of these signals can lead to anergy or cell death. CD28 has recently been described as an important accessory signal for T cell activation. Disruption of CD28 engagement to its ligand CD80, with monovalent anti-CD28 Fab fragments results in hypo-responsiveness to secondary stimulation by a specific alloantigen (Harding et al. 1992). Similar results occur if CD80 negative APCs are used to induce alloantigen responses by $CD4^+$ T cells (Boussiotis et al. 1993). Monoclonal antibodies against CD28 in conjunction with PMA can induce proliferation and IL-2 production in T cells. This signalling pathway is cyclosporin resistant but utilises the IL-2/IL-2R pathway and can induce dramatic increases in T cell responses (Linsley and Ledbetter, 1993; van Seventer et al. 1992).

Unlike transmembrane signals through CD2 or CD11a/CD18, CD28 does not employ the same second messengers as the TCR/CD3 complex. In addition, cell adhesion does not appear to play a major role in CD28 function (Freeman et al. 1993). CHO cells transfected with CD80 can stimulate T cells in the presence of CD3 MAbs. In addition, CD80 transfected cells can provide stimulation for $CD4^+$ T cells to alloantigen. Surprisingly, the CD80 molecule that activates CD28 does not have to be expressed on the same stimulator cell surface as the MHC molecule (Spits, 1987).

Since the addition of cytokines had no significant effect on T cell proliferation against $DR1^+$ TFCL, it was possible that a membrane bound co-stimulus was involved.

EXPERIMENTAL DESIGN

The following formula was used to calculate the effect of autologous APCs compared to control cell stimulation.

$$\text{Stimulation index} = \frac{\text{test } [^3\text{H}]\text{TdR - background}}{\text{control } [^3\text{H}]\text{TdR - background}}$$

where the control = response without autologous APC. Background is the response in the presence of cytokines with FCL. An SI>2.5 was considered to be significant.

The monoclonal antibody CD28 was titrated on PHA stimulated PBMC using flow cytometry to determine the optimal concentration of antibody. One of four methods was used to assess the co-stimulatory ability of CD28 MAb, each of which is described below.

a.) Flat-bottomed 96 well plates were coated with 1μg/ml GαM and incubated for 1 hour at 4°C. The wells were then washed three times in serum free HBSS before incubation with 1μg/ml of CD28 MAb for a further hour at 4°C. Finally, the plates were washed three times in serum free HBSS before being set up in a primary MLR.

b.) Flat bottomed plates were incubated with 1μg/ml of CD28 MAb as above in the absence of GαM.

c.) Soluble MAb CD28 was added directly to the wells (1μg/ml) at the start of the MLR.

d.) PBMC or CD45R subsets at a concentration of 10^6 cells/ml were incubated with 1μg/ml of CD28 MAb at 37°C for 30mins. Cells were then washed thoroughly in serum free HBSS before being added to the flat-bottomed 96 well plates.

Primary MLRs were set-up as previously described (see Section 2-5), following pre-treatment with CD28 by one of the above methods.

The Mann-Whitney non parametric two tailed U-test was used to compare T cell proliferation on the data from five experiments for both APC and CD28 studies. Proliferation by responder subsets against the control cell line FCL were subtracted from each donor prior to log transformation of $[^3\text{H}]$ TdR uptake for CD28 studies.

RESULTS.

Five HLA-DR1 negative individuals were stimulated with either TFCL or $DR1^+$ LCL in the presence or absence of purified autologous monocytes and prolfieration was measured by $[^3\text{H}]$TdR uptake.

Responses are represented as a percentage proliferation against the control cell line, FCL (Fig. 6-1). Responses by both $CD4^+CD45R^+$ populations against $DR1^+$ $CD58^+$ TFCL were similar to $DR1^+$ $CD54^+$ TFCL (data not shown). Collective analysis of all five donors revealed that proliferation by $CD4^+RA^+$ T cells against $DR1^+$TFCL, $DR1^+CD58^+$ TFCL and $DR1^+$ LCL, were significantly increased by the addition of autologous monocytes. Stimulation by $DR1^+$ TFCL and $DR1^+$ LCL was similarly increased in both CD45R subsets. Responses against $DR1^+CD58^+$ TFCL were enhanced by autologous monocytes although the increase was not statistically significant (data not shown). When $CD4^+RA^+$ and $CD4^+RO^+$ T cell responses against TFCL and $DR1^+$ LCL in the presence or absence of autologous monocytes were compared, no statistically significant differences were detected. Proliferation by RA and RO subsets against $DR1^+$ $CD58^+$ TFCL was similarly (data not shown). Although the presence of autologous APCs greatly enhanced proliferation by either CD45R subset to TFCL (between two and five fold), and two fold greater with $DR1^+$ LCL induced proliferation there was no selective effect on either. $DR1^+$ LCL continued to elicit ten fold greater responses against TFCL and five fold greater than $DR1^+CD58^+$ TFCL in the presence of APC. Responses against $DR1^+CD54^+$ TFCL were similar to $DR1^+CD58^+$ TFCL (data not shown).

Monocytes clearly provide a signal that is absent in TFCL stimulated responses. It is possible that the CD28-CD80 pathway, an important co-stimulus in T cell activation may be absent. To investigate this, T cell subsets were stimulated with TFCL as before in the presence or absence of CD28. Since the transfected cell lines were all adherent, plate-bound CD28 would be unable to effectively co-stimulate T cells in this model. An alternative method of presenting CD28 was required. In order to assess the most efficient method of co-stimulating T cells with CD28, four methods were compared in a situation where CD28 co-stimulation has previously been demonstrated (see Experimental Design in this Chapter). Comparison of PBMC proliferation against $DR1^+$LCL in the presence or absence of CD28 MAb is shown in Fig. 6-2. Pre-treatment of the responder population with CD28 MAb for 30 mins at 37°C was as effective as crosslinking with GαM. Pre-treatment was therefore selected as the preferred method to use in co-stimulation studies thereafter.

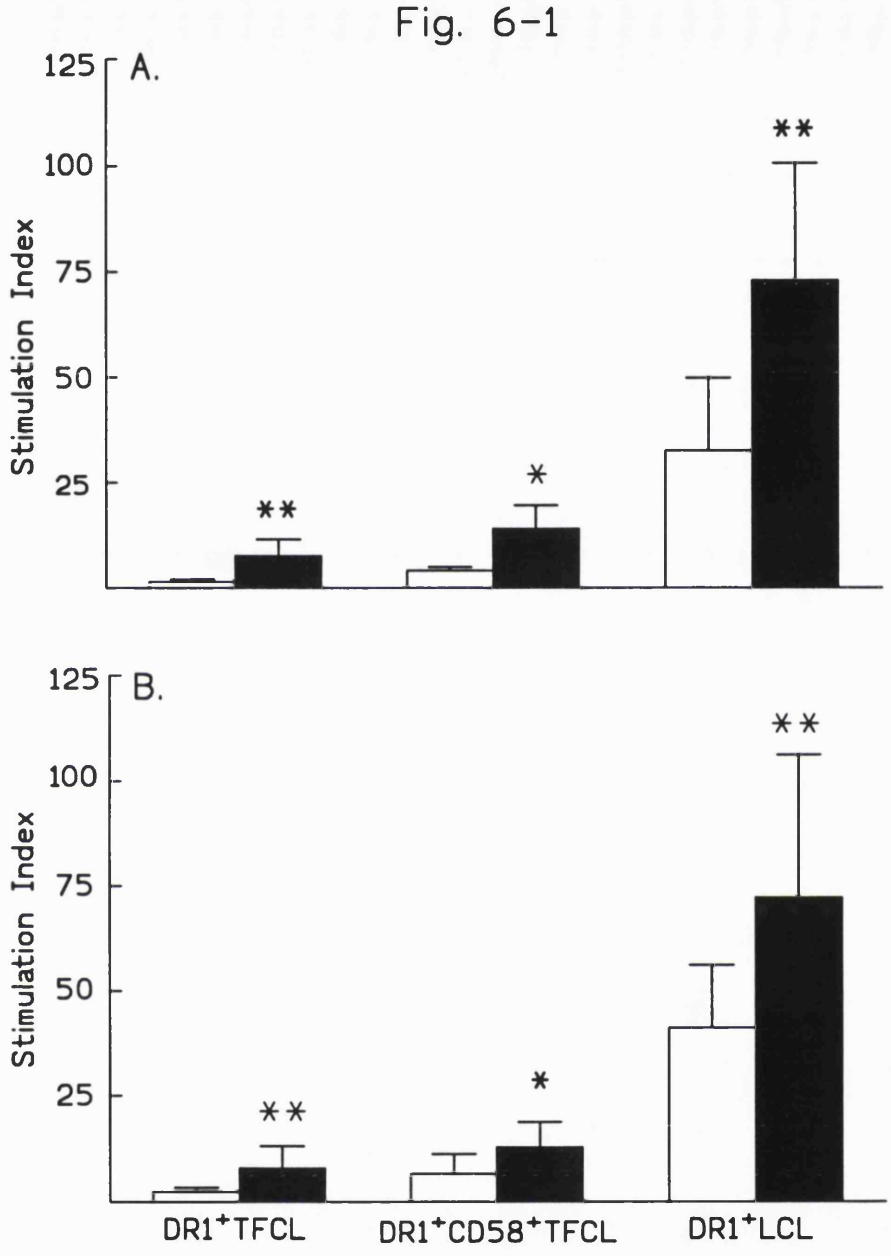

Figure 6-1: The effect of autologous APC on alloantigen responses.
A) RA^+ and B.) RO^+ T cell responses against $DR1^+$ TFCL, $DR1^+CD58^+$ TFCL and $DR1^+$ LCL in the presence (solid bars) or absence (open bars) of autologous APC. The calculation to obtain the increase compared to the control FCL response is described in Section 6-2. Data represents the mean of five separate experiments.* $p<0.05$, ** $p<0.01$ using the Mann-Whitney non-parametric two tailed U-test, calculated on the software described in Section 2-5.

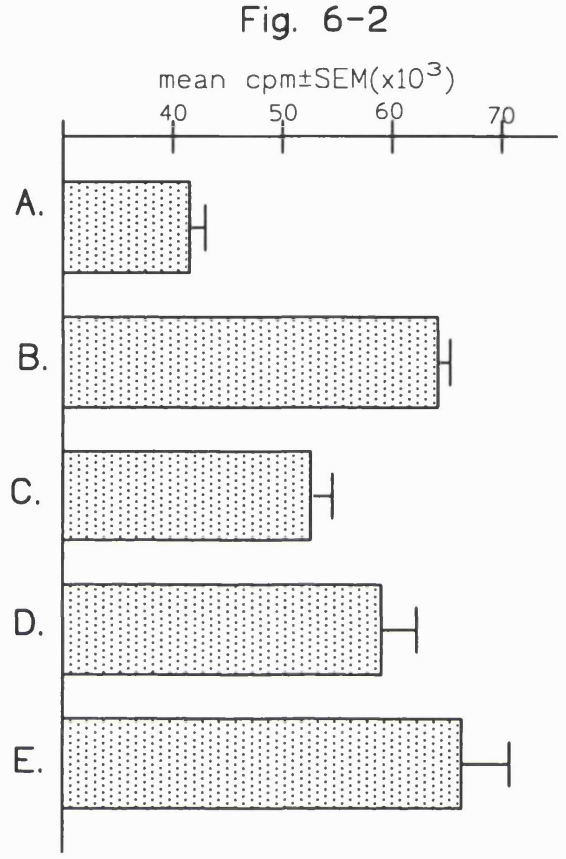

Figure 6-2: Comparison of CD28 pre-treatment on alloresponses.

Freshly isolated PBMCs were stimulated with $DR1^+$ LCL (A) alone; (B) CD28 + GαM; (C) plastic adhered CD28 ; (D) soluble CD28 or APC pulsed with CD28 (as described in Section 6-2).

Bars represent the mean cpm ± SEM ($x10^3$) of responses from two separate HLA-DR1 negative donors.

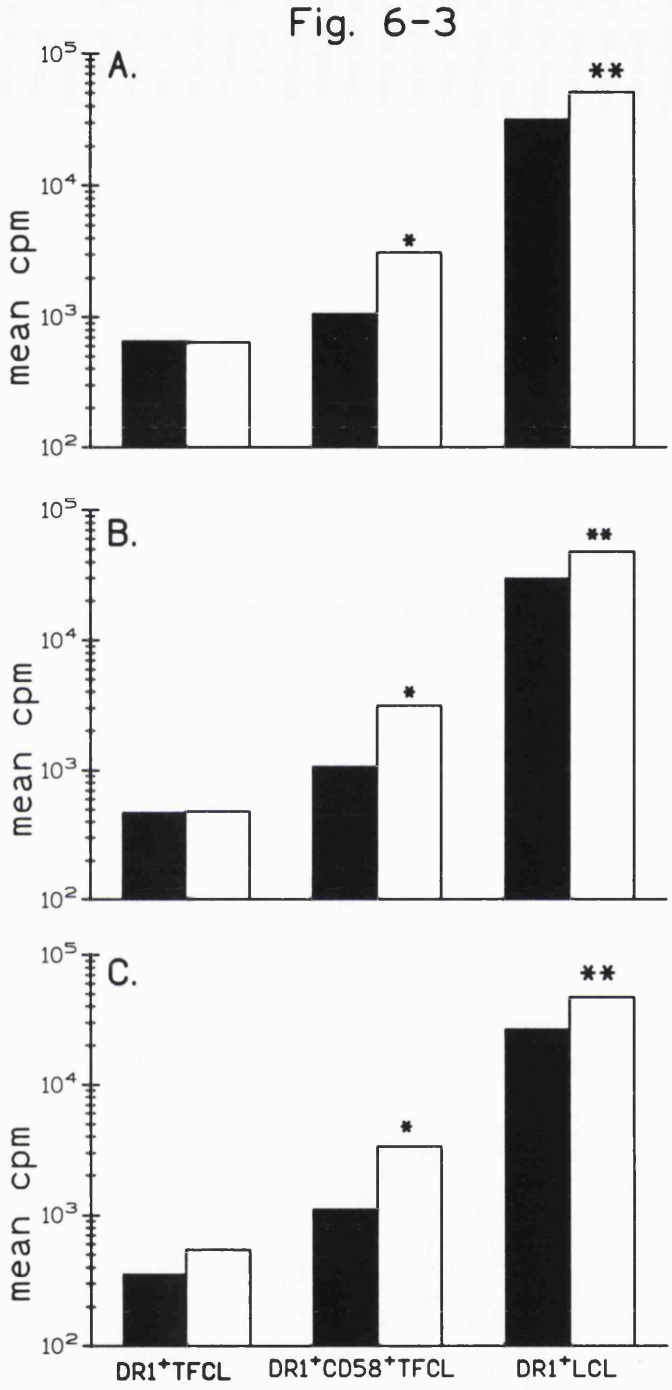

Figure 6-3: The effect of CD28 on T cell responses to alloantigen.

Stimulation of A.) PBMC, B.) RA^+ and C.) RO^+ T cells in the absence (solid bars) or presence (open bars) of the CD28 MAb. Bars represent the mean of four separate HLA-DR1 negative donors. See Appendix 6-II for individual responses.
* $p<0.05$ ** $p<0.01$

PBMC and CD45R subsets from four DR1 negative individuals were stimulated with TFCL or $DR1^+$ LCL in the presence or absence of a CD28 MAb. Collective analysis of all four donors showed that the addition of CD28 MAb to PBMC or either $CD4^+$CD45R subsets augmented proliferation against $DR1^+$ LCL ($p<0.01$) and $DR1^+$ $CD58^+$ TFCL ($p<0.05$) but had little effect on $DR1^+$ TFCL (Fig. 6-3). No differences between $CD4^+$CD45R populations were detected.

DISCUSSION.

Peripheral blood DCs have been shown to be one of the most potent APCs in both humans and mice, more so than either monocytes or B cells. Despite this, monocytes are the most widely used as the source of APCs from peripheral blood for *in vitro* studies, since peripheral blood DCs are difficult to purify with a tendency to alter in phenotype and function once isolated. Nonetheless, monocytes can function as effective APCs in an MLR and were chosen as the source of autologous APCs in this instance. Purity of the autologous APCs was vital to ensure that this was the only cell subset being added back to the purified CD45R T cell subsets. Purification through the G10 sephadex column proved to be most efficient for removing monocytes from peripheral blood.

When the expression of various surface markers were compared, CD54 and CD58 expression on autologous monocytes were comparable to expression on the $DR1^+$ LCL (Table 2-III). Expression of HLA-DR was approximately 2.5 fold lower on autologous monocytes, although levels were at a sufficient level to act as APCs, with levels of HLA-DR expression similar to TFCL.

The presence of autologous monocytes significantly enhanced responses of both $CD4^+$ CD45R subsets to TFCL, showing that autologous monocytes can provide additional signal components absent from TFCL stimulation alone. $CD4^+$CD45R T cell subsets benefited equally from the presence of autologous monocytes. This was unexpected since previous studies have suggested that naive T cells require professional APC and are more dependent upon co-stimuli when -stimulated with CD3 MAb or CD2 MAbs (Damle et al. 1992; Ronchese and Hausmann, 1993; Larsen et al. 1992). Our studies showed no such differences, as did similar studies by Thomas and Merkenschlager (Thomas et al. 1993; Merkenschlager et al. 1991).

Responses against $DR1^+$ LCL were also enhanced by the presence of autologous monocytes. The enhancement seen in these experiments additional effects by could be due to a.) an autologous MLR (aMLR); b.) increasing the number of APCs augments proliferation; c.) presentation by the direct and indirect pathway or d.) monocytes may provide additional co-stimuli. Although aMLRs were detectable, the use of an the FCL control cell line responses as a baseline eliminates this problem. Careful titration of cell numbers in the initial experiments meant that option b.) is also unlikely. The final explanation for augmented proliferation by monocytes in $DR1^+$LCL induced activation is the employment of the alternative antigen presenting pathway. Up until now, all studies in this project have used the direct pathway. The introduction of autologous monocytes would involve alloantigen from the TFCL or LCL being processed and presented by the autologous monocytes. This pathway may be more effective since the APCs would be recognised as 'self'. Autologous monocytes clearly provide an activation signal that is lacking in TFCL activated cultures. Earlier studies have shown that this is unlikely to be due to a single cytokine acting alone although combinations of cytokines cannot be ruled out.

Ligation of CD28 with its ligands CD80 (B7-1) or CD86 (B7-2), generates a co-stimulatory signal that is independent of the TCR/CD3 signal, blocking of this signal induces T cell non-responsiveness (Harding et al. 1992). RA^+ T cell are less responsive to CD3 MAb than RO^+ T cells (Byrne et al. 1988; de Jong et al. 1992), these differences cannot be overcome by the addition of MAbs against CD28 or cells cotransfected with CD32, the human FcγRII and CD80. This suggests that naive cells have a higher threshold for CD28 co-stimulation than memory cells. If a similar experiment is carried out using alloantigen as the stimulus, CD45R T cell subsets benefit equally from the presence of CD28 MAb indicating that the differences described in the previous study using $CD32^+$ $CD80^+$ TFCL and CD3 as the stimuli, were describing a difference in the threshold for CD3 stimulation and not CD28. The presence of CD28 MAb augments the responses by all responder populations tested against $DR1^+$ LCL. Clearly, the CD28 stimulation pathway has an important role in T cell responses against alloantigen whether or not it is involved in CD3 stimulation of naive cells.

Other studies however, have been able to demonstrate increased T cell proliferation in the presence of CD28 MAb with $DR4^+$ transfected CHO cells pulsed with SEA (Sansom et al. 1993).

This augmentation could also be induced if the TCR signal and the co-stimulus are on separate cell surfaces. Discrepancies between these studies and our own may be due to the level of stimulation induced by SEA and alloantigen.

Stimulation of T cell populations by $DR1^+$ TFCL were not augmented by the addition of CD28 MAb. A lack of adhesion between the T cell and the APC is the likely cause of this since adhesion is not considered a major function of CD28. The primary function of the CD28-B7 pathway is co-stimulation. Since the $DR1^+$ TFCL does not express any human adhesion molecules, contact between the two cells may not be sufficient to induce proliferation. Alternatively, CD28 may not function effectively when it is plate bound as opposed to the surface of the APCs.

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CHAPTER 7.

PRIMING OF T CELLS WITH DR1$^+$TFCL AND DR1$^+$LCL.

INTRODUCTION.

The engagement of the TCR can lead to either cell proliferation, non-responsiveness or, in some circumstances, cell death (Jenkins and Schwartz, 1987; Bretscher, 1992; Jenkins, 1992; Liu and Linsley, 1992; Janeway and Bottomly, 1994). The outcome is primarily dependent on the engagement of the TCR, the presence of a co-stimulus and the type of signals they can induce. Blocking accessory cell interactions such as CD2-CD58 or CD11a/CD18-CD54 with MAbs during primary stimulation of alloreactive T cells can block proliferation and lead to a state of unresponsiveness (Sanders et al. 1988; Springer, 1990; Gerli et al. 1993; Chavin et al. 1994). This can be overcome by the presence of IL-2 upon re-challenge or prevented altogether by the addition of IL-2 during the initial stimulation (Boussiotis et al. 1993; Schwinzer et al. 1994).

The accessory pathways CD11a-CD18/CD54 and CD2/CD58 provide adhesion in addition to co-stimulatory pathways for T cell activation (Hunig, 1985; Rothlein and Springer, 1986; Beyers et al. 1989; Littman et al, 1989; Schraven et al. 1990; Pardi et al. 1992a; Pardi et al. 1992b; Haverstick and Gray, 1992). The DR1$^+$ LCL used for allostimulation, expresses a wide range of accessory molecules involved in adhesive contacts between the T cell and the APC as well as inducing co-stimulatory signals (Table 7-1). DR1$^+$ TFCL however, does not express any human accessory molecules signals and induces low level proliferation suggesting that engagement of the TCR through alloantigen expressed on the TFCL may lead to non-responsiveness or cell death. CD4$^+$ T cells have previously been shown to proliferate against DR7$^+$ TFCL co-transfected with either CD80 or CD54. Although both molecules provided costimulation for CD4$^+$ T cells, only CD80 induced IL-2 production (Boussiotis et al. 1993). The same study investigated the roles of CD54 and CD80 in the induction of tolerance. Priming of CD4$^+$ T cells with either DR7$^+$ TFCL or DR7$^+$CD54$^+$ TFCL induced tolerance with no significant proliferation upon restimulation with either DR7$^+$, DR7$^+$CD54$^+$ or DR7$^+$CD80$^+$ TFCL. However, cells primed in this manner were able to respond to either a third party alloantigen, recombinant IL-2 or PMA plus an ionophore.

Table 7-I: Surface expression of accessory molecules by $DR1^+$ LCL.

Ag	Other name	Density of Expression*
CD19	-	++
CD11a	LFA-1α	+++
CD28	-	+/-
CD29	-	+
CD38	-	+
CD45RA	-	+
CD45RB	-	+++
CD49d	VLA-4	++
CD50	ICAM-3	+++
CD54	ICAM-1	+++
CD58	LFA-3	+
CD62L	L-selectin	++
CD80	B7/BB1	++
CD102	ICAM-2	+
HLA-DR	-	++++

$DR1^+$ LCL is a homogenous cell line, all molecules are expressed on >98% of the population. * The code used for density of expression is as follows: -/+ = 0-50; + = 50 - 100; ++ = 100-200; +++ = 200-300 and ++++ = >300.

Priming of $CD4^+$ T cells with $DR7^+CD80^+$ TFCL did not induce tolerance to $DR7^+$, $DR7^+CD54^+$or $DR7^+CD80^+$ TFCL. From these studies, Boussiotis *et al* concluded that CD54 and CD28 utilise different costimulatory pathways and also differ in the ability to prime naive T cells and induce tolerance to alloantigen (Boussiotis et al. 1993).

The lack of human accessory molecules is not the only explanation for the reduced levels of proliferation against TFCL. The process of transfecting human HLA-DR1 into murine cells could modify the structure of HLA-DR1. In this instance, the murine cell would be expressing defective HLA-DR1 that would be unable to present antigen to human T cells or interact with the TCR. By priming T cells with $DR1^+$ LCL and rechallenging these cells with $DR1^+$ TFCL, the ability of DR1 specific cells to recognise and respond to $DR1^+$ TFCL could be tested.

EXPERIMENTAL DESIGN

Whole PBMCs were incubated in a primary MLR for five days with either the $DR1^+$ LCL or the $DR1^+$ TFCL in a $25cm^3$ culture flask. On day 6, cells were washed in HBSS supplemented with 5% FCS and resuspended in fresh culture medium at a concentration of 10^6cells/ml for a further 24 hrs. On day 7, cells were rechallenged for a further five days by either of the stimuli used in the primary MLR and third party stimulators $DR11^+$ TFCL or a $DR11^+$ LCL. Proliferation was assessed by a 4 hr pulse with [^{3}H]TdR on days 3, 5, 6, 7, 9 and 11. Viability was assessed on the same days by trypan blue exclusion.

RESULTS.

Freshly isolated whole PBMC from normal DR1 negative individuals produced low levels of proliferation against $DR1^+$ TFCL in the primary MLR in comparison to high levels of proliferation against $DR1^+$ LCL (see Fig. 3-3). To investigate the possibility that low proliferation was caused by the expression of defective HLA-DR1 on the TFCL, PBMCs were primed with either $DR1^+$ TFCL or $DR1^+$ LCL rechallenged with the same stimuli (Fig. 7-1 to 7-5).

Priming of PBMCs with $DR1^+$ TFCL produced low levels of proliferation as expected If these cells were rechallenged with the same stimulus, proliferation was 10-fold greater than in the primary response and increased progressively up to and including day 14. Re-challenging the $DR1^+$ TFCL primed cells with $DR1^+$ LCL produced a typical secondary response curve in four out of five of the individuals tested.

No proliferation was observed in the secondary responses stimulated with either $DR11^+$ TFCL or $DR11^+$ LCL (Figs. 7-1 to 7-5).

Cells primed with $DR1^+$ LCL for five days and re-challenged with the same stimulus, produced a typical secondary response that peaked three days earlier than the primary response between days 1 and 3 (Fig. 7-1B to 7-5B). These types of primary and secondary responses occurred in all five individuals tested. If $DR1^+$ LCL primed cells were rechallenged with $DR1^+$ TFCL, secondary responses similar to rechallenge with $DR1^+$ LCL were observed in four out of the five individuals tested. No proliferation was observed if cells were re-challenged with the control cell lines $DR11^+$ TFCL or $DR11^+$ LCL (Fig. 7-1B to 7-5B).

Clearly, primary stimulation with $DR1^+$ TFCL does not lead to alloantigen specific hypo-responsiveness or cell death when alloantigen was presented in the manner described here. Table 7-I shows the wide variety of accessory molecules expressed on the $DR1^+$ LCL. $DR1^+$ TFCL on the other hand only expresses human MHC class II that has been transfected into the cell artificially. HLA-DR is expressed at levels that are approximately 3-fold lower than HLA-DR naturally expressed on the $DR1^+$ LCL (see Chapter 2-4). To investigate whether changes in surface antigen expression occurred with alloantigen stimulation, freshly isolated PBMC were primed for five days with either $DR1^+$ TFCL or $DR1^+$ LCL and levels of activation antigen expression were assessed (Table 7-II & Fig. 7-6). The percentage of T cells expressing HLA-DR, RO and CD25 increased when $DR1^+$ TFCL was the stimulus. In contrast, the majority of activation antigens tested, with the exception of CD38, increased when $DR1^+$ LCL was the stimulus. Analysis of CD45R isoform expression during this period showed that a percentage of $DR1^+$ TFCL stimulated cells did switch from RA^+ to RO^+ during this period. The percentage of T cells switching CD45R isoform in $DR1^+$ LCL stimulated cultures was greater, with a larger percentage of T cells expressing RO.

To investigate changes in surface molecule expression further, whole PBMCs were were incubated with either $DR1^+$ LCL or TFCL in a primary MLR and stained for activation markers on days 0, 1, 3 and 5 (Figs 7-6 & 7-7). The percentage of T cells expressing various activation antigens clearly varied during priming.

Cells stimulated with $DR1^+$LCL showed progressive increases in the percentage of cells expressing HLA-DR, RO, CD54 and CD58. The percentage of cells expressing RA decreased with increasing RO expression, which correlated with the increased number of cells expressing both CD45 isoforms. CD4, CD11a and CD38 expression remained constant over the period.

Table 7-II: Changes in activation molecules on alloantigen stimulated PBMC.

Antigen	Resting PBMCs	$DR1^+$TFCL Stimulated cells*	$DR1^+$LCL Stimulated cells*
HLA-DR	30±4	42±9	63±5
CD45RA	46±4	30±5	32±9
RO	30±5	52±12	47±4
RA^+RO^+	51±3	39±7	23±6
CD2	65±5	72±4	46±4
CD25 (IL-2R)	13±2	11±3	11±6
CD26	36±12	41±5	21±3
CD38	20±7	32±9	18±5
CD54	51±2	12±8	27±9
CD58	43±7	61±11	39±8
CD4	39±6	33±9	24±5
CD69	4±2	9±4	1±3
CD11a	71±4	65±6	43±5

Numbers represent the mean ± SEM percentage of T cells from five individual experiments. T cells were gated using CD3 and TCR MAbs and the percentage of T cells expressing each specified antigen was determined from this gate. Resting cells are freshly isolated PBMCs stained for activation antigens on day 0, the remaining columns represent the percentage of gated T cells following five days stimulation with either $DR1^+$TFCL or $DR1^+$LCL. RA/RO are the percentage of RA^+RO^+ double positive T cells.

Fig. 7-1

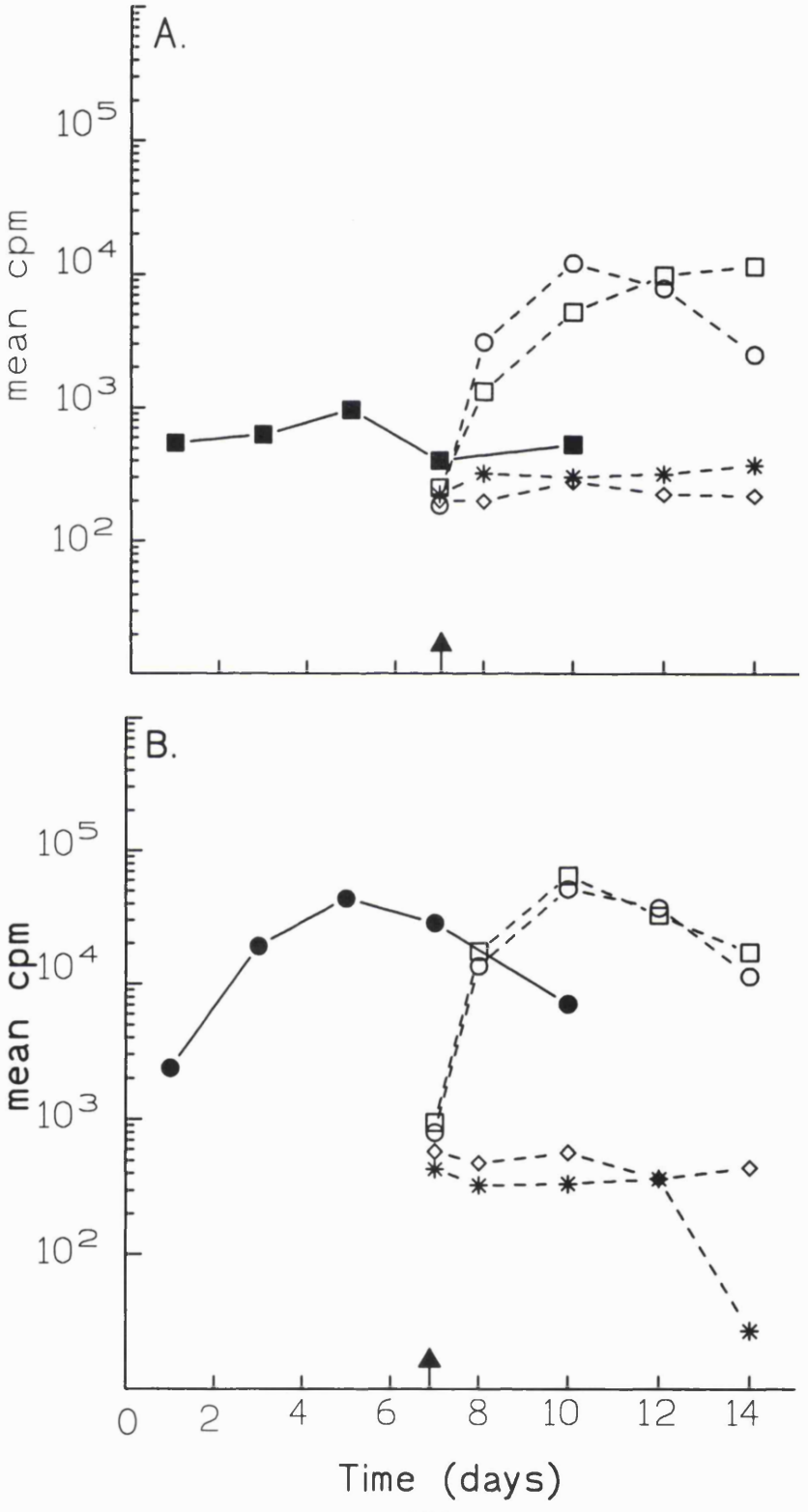


Fig. 7-2

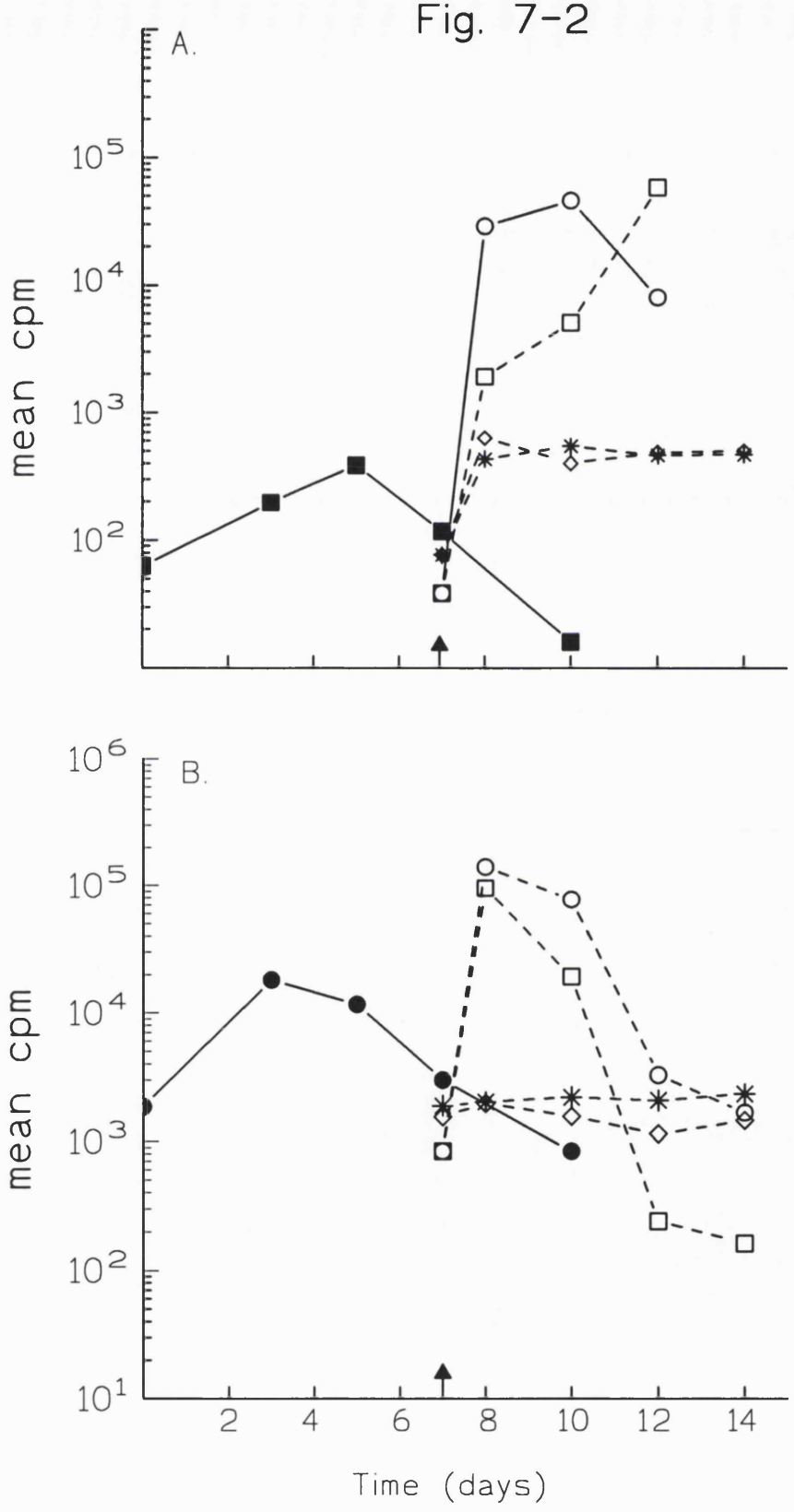


Fig. 7-3

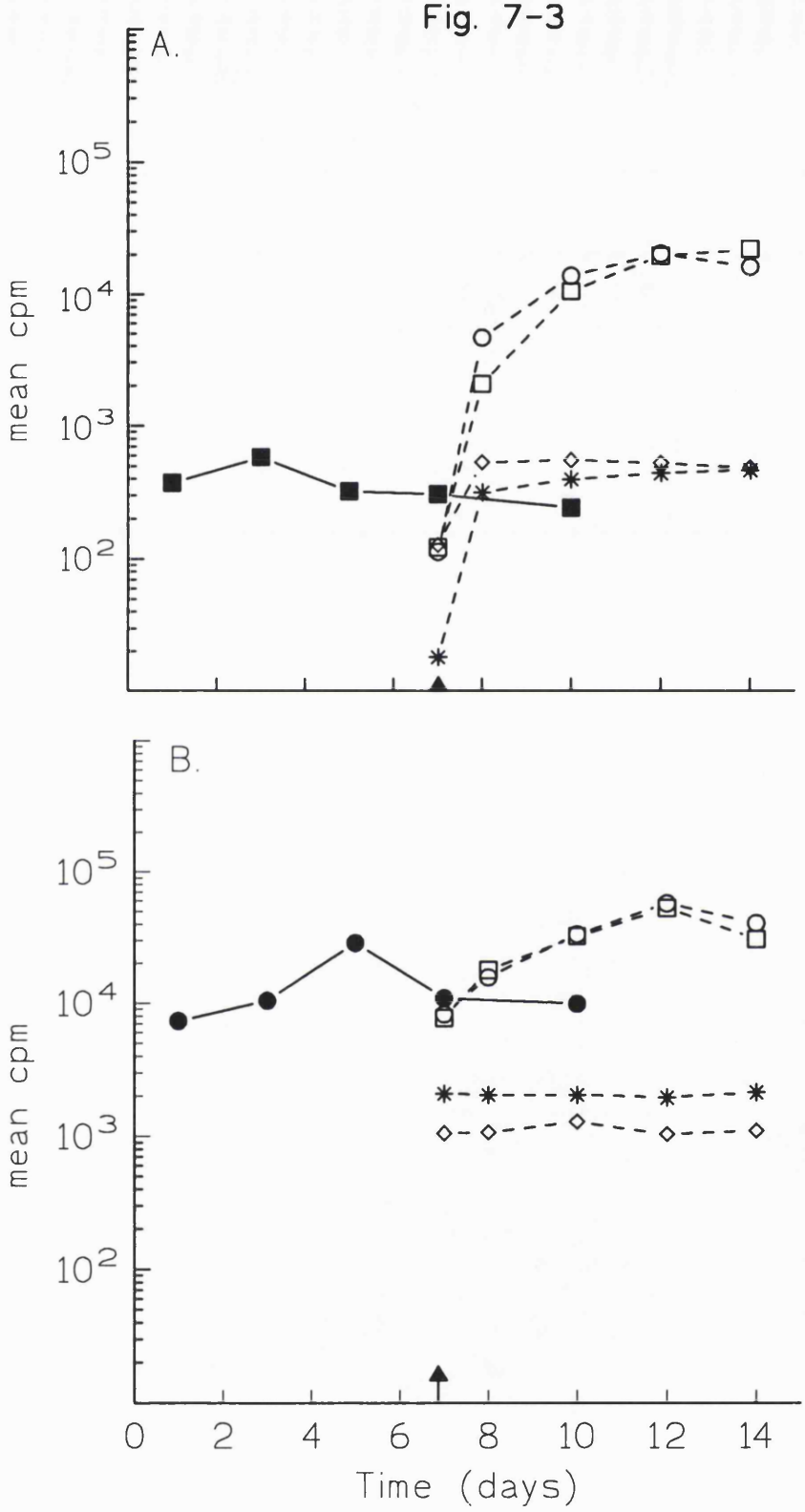
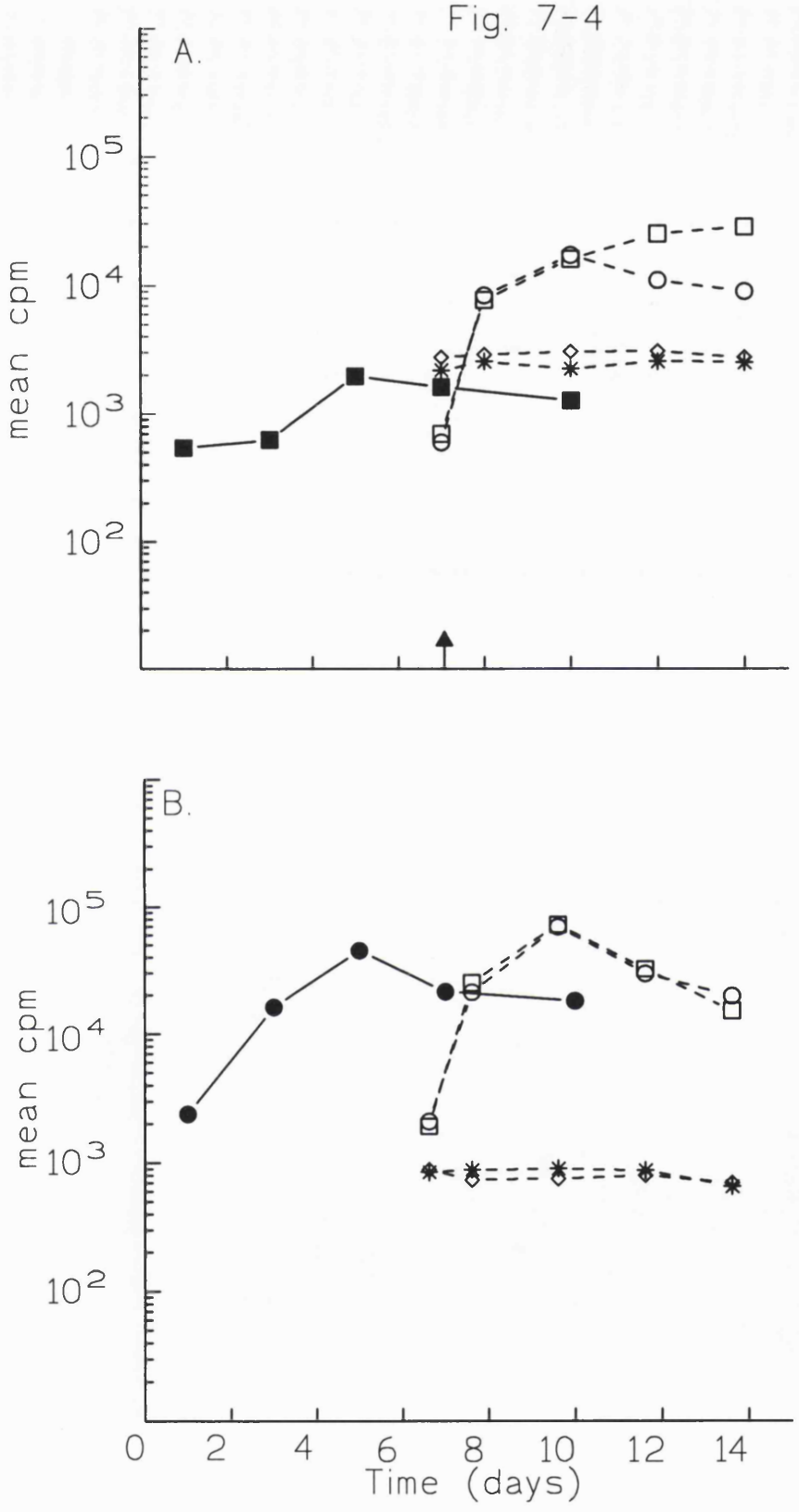


Fig. 7-4



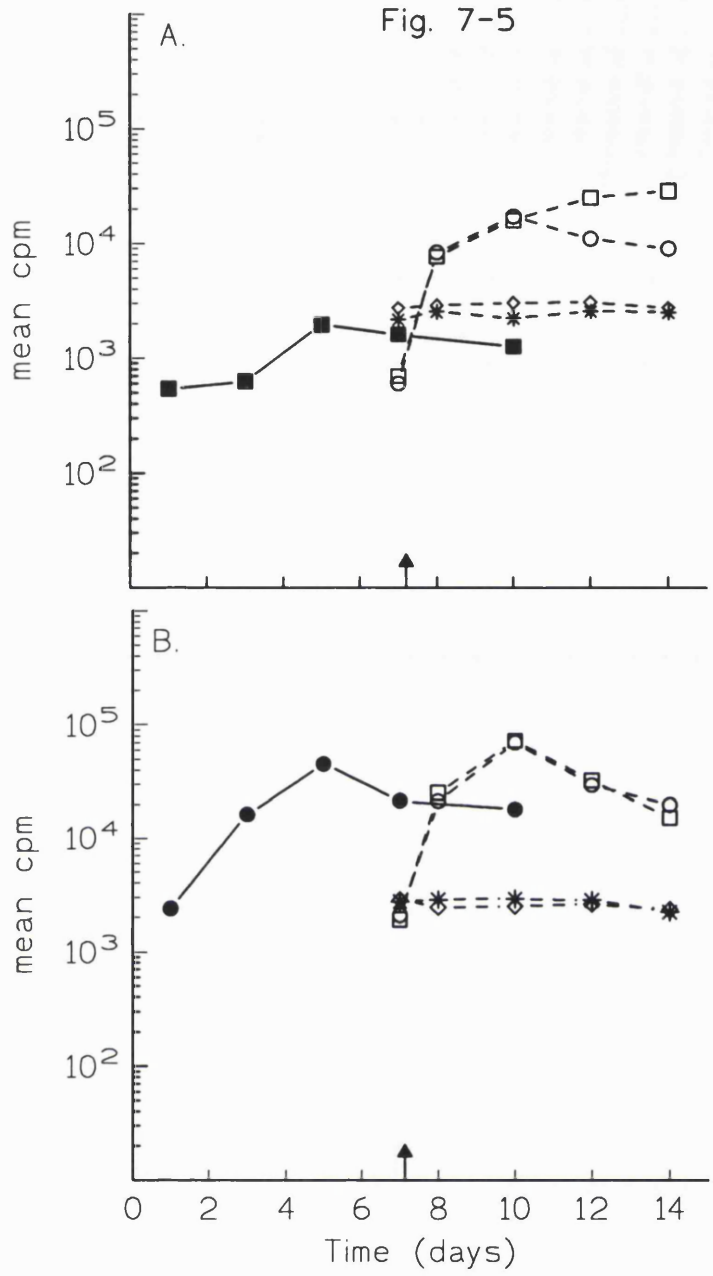


Figure 7-1 to 7-5: Exposure of PBMCs to DR1$^+$TFCL leads to priming.

The effect of DR1$^+$ TFCL and DR1$^+$ LCL on stimulating primary and secondary MLRs. PBMCs were washed after a primary challenge and 'rested' for one day then re-challenged with allostimulatory cells. A.) Cells stimulated in a primary MLR with DR1$^+$ TFCL (■-■) and rechallenged with either the DR1$^+$ LCL (❍--❍), DR1$^+$ TFCL (□--□), DR11$^+$ TFCL (✧--✧) or the DR11$^+$ LCL (∗-- ∗) B.) cells primed with the DR1$^+$ LCL (●-●) legend as in A.). Arrow (↑) rechallenge on day 7, solid lines represent primary stimulation and dotted lines represent secondary stimulation. Figures 1 to 5 represent five different individual donors.

Fig. 7-6

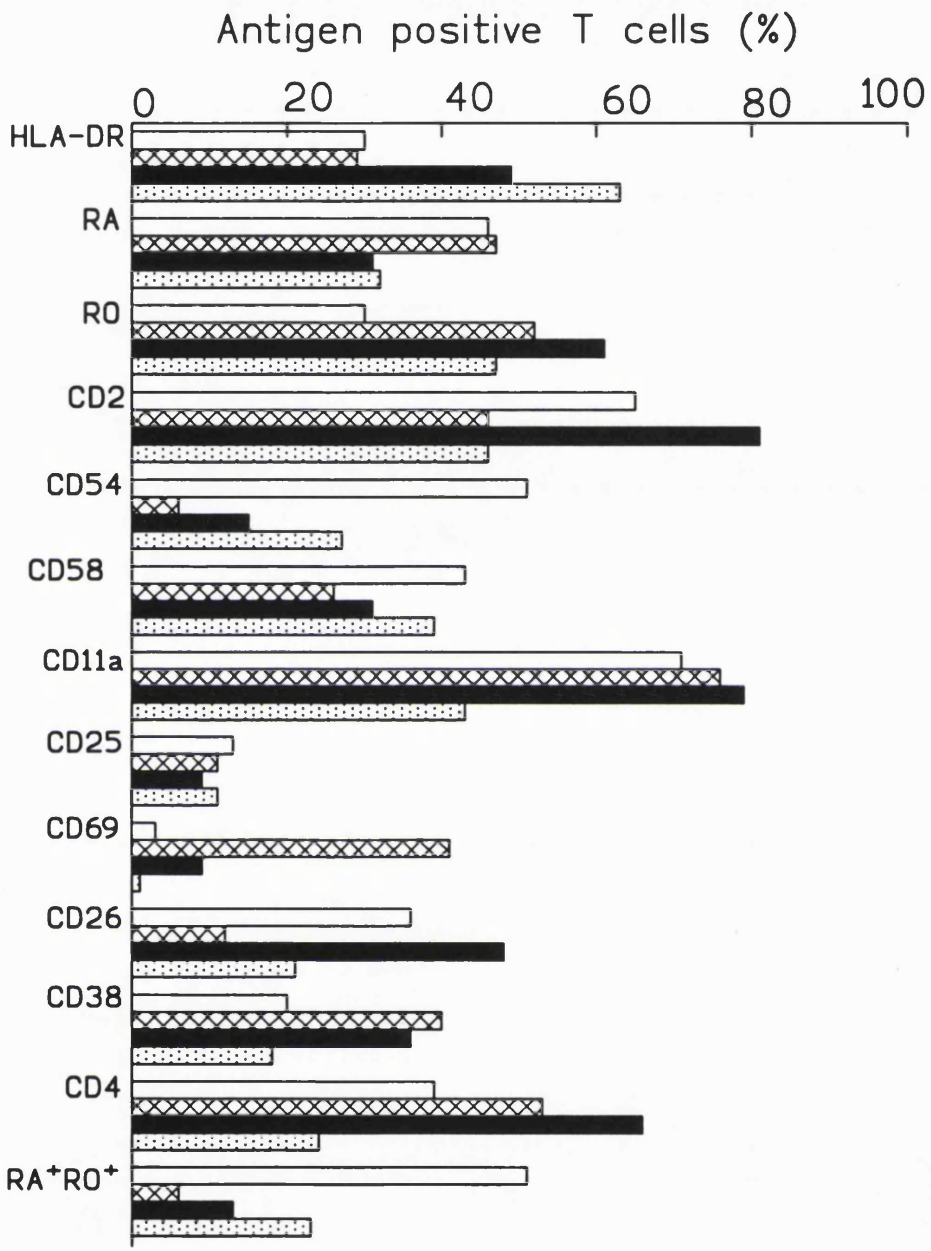


Figure 7-6: Activation antigen expression on PBMCs stimulated with DR1$^+$ LCL over five days.

Open bars = day 0 (resting cells); hatched bars = day 1; solid bars = day 3 and speckled bars = day 5.

Fig. 7-7

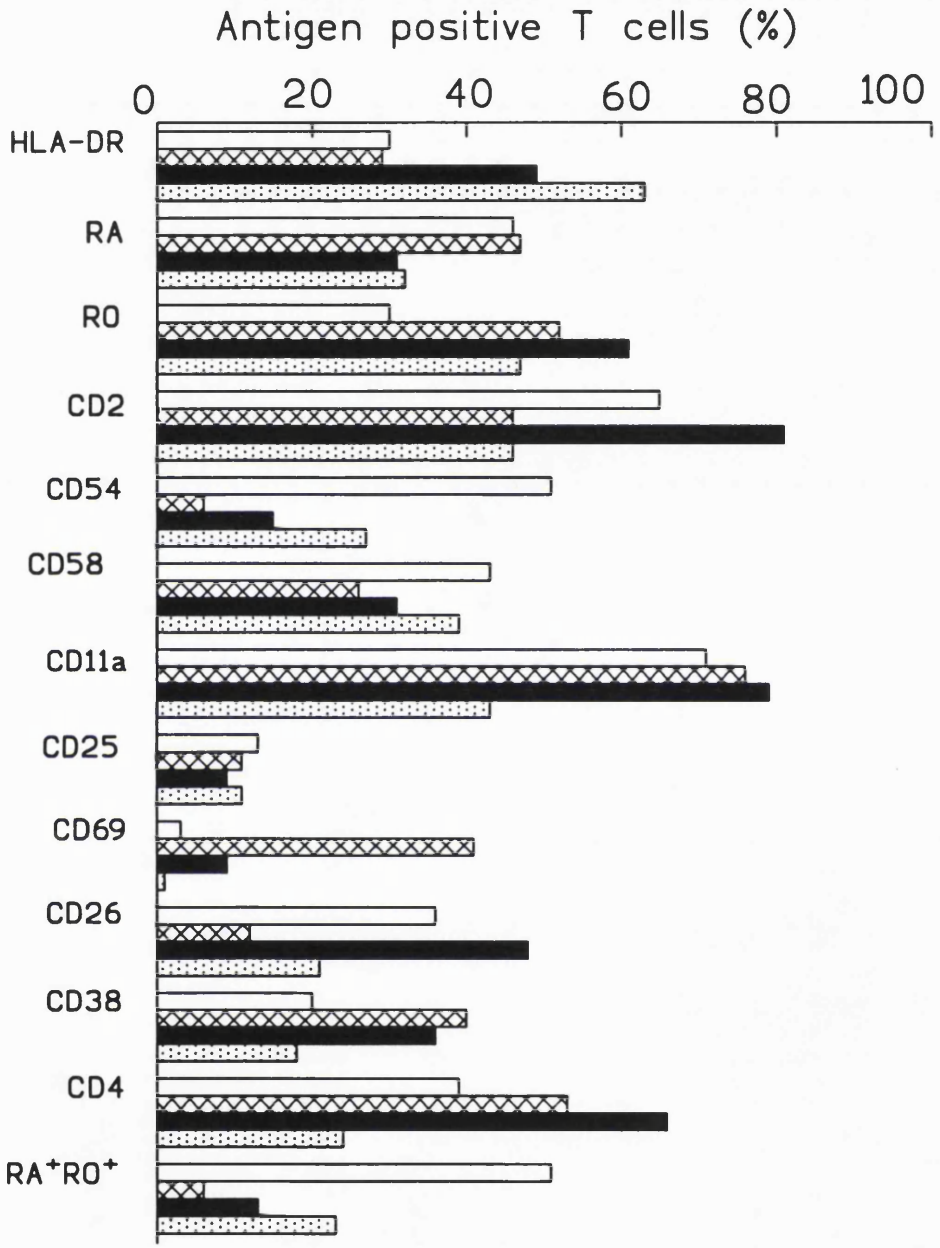


Figure 7-7: Activation antigen expression of PBMCs stimulated with DR1$^+$ TFCL over 5 days.

Bars as in Fig. 7-6.

In some cases, the percentage of cells expressing a particular antigen decreased initially and increased thereafter, such antigens included CD54 and CD38. CD69 and CD26 increased initially and decreased thereafter.

The analysis of cells stimulated with $DR1^+$ TFCL showed similar patterns of expression to $DR1^+$ LCL for HLA-DR, CD11a and CD25, although the all of the antigens were expressed on a lower percentage of T cells. The percentage of cells expressing CD2, CD54 and CD26 were not affected by $DR1^+$ TFCL stimulation. However, the early activation markers CD69 and CD26 were unaffected. A decrease in the percentage of cells expressing RA did occur and paralleled the increased number of cells expressing RO, although as with the majority of other activation antigens tested, the percentage of cells was lower than in $DR1^+$ LCL stimulation.

DISCUSSION.

T cells primed against HLA-DR1 are able to recognise and respond to $DR1^+$ TFCL upon rechallenge. This excludes the possibility that transfection of human HLA-DR1 into murine fibroblast cells induces changes in the structural conformation of the MHC molecule to such an extent that TCR are unable to bind and respond to alloantigen presented in this fashion. This may be a result of the continuation/magnification of the primary response. However, rechallenge of $DR1^+$ TFCL primed cells with $DR1^+$ LCL is not an amplification of a primary MLR since some individuals showed proliferative responses with characteristics of a secondary response.

Whole PBMCs were used to produce HLA-DR1 'memory' T cells since priming with purified $CD4^+$CD45R T cell subsets was unsuccessful. Stimulation of CD45R separated subsets with $DR1^+$ TFCL led to poor harvests of viable cells. Memory cells to HLA-DR1 expressed on $DR1^+$TFCL could recognise and respond to the same molecule upon rechallenge in the apparent absence of accessory molecules.

Priming with $DR1^+$ TFCL is also HLA-DR1 specific as rechallenge with $DR11^+$TFCL or $DR11^+$LCL did not induce proliferation. The weak rise in primary responses against $DR1^+$ TFCL indicates that if $DR1^+$ TFCL are able to induce priming of T cells in the absence of high levels of proliferation, the co-stimulus must be either IL-2 released by the T cell subsets themselves or a murine homologue of accessory molecules such as CD58 or CD54 on the murine fibroblast. Priming with $DR1^+$ LCL and rechallenge with either $DR1^+$ TFCL or $DR1^+$ LCL led to strong secondary responses. These priming studies indicate that expression of co-stimulatory molecules is vital to the production of primed T cells but secondary responses can be induced in the absence of accessory signals. There is also the possibility that $DR1^+$ TFCL could help specific cells to survive in culture which is augmented by the secondary MLR.

Assessment of activation molecule expression on responder cells during stimulation showed that $DR1^+$ TFCL can induce changes in MHC class II, and other activation molecules in the absence of high level proliferation. A change in phenotype from RA^+ to RO^+ is also induced. $DR1^+$ LCL stimulated cells behave in a similar manner to T cells described previously. Stimulation by alloantigens induced significant changes in CD45 isoform expression with increases in the percentage of RO^+ T cells and a decrease in RA^+ T cells as expected.

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CHAPTER 8

GENERAL DISCUSSION & CONCLUDING REMARKS

RA^+ and RO^+ T cells have the same activation requirements when stimulated with alloantigen. Provision of even the minimal signaling requirements necessary for T cell activation cannot differentiate between these two T cell populations. Previous studies have shown that CD45R populations respond equally to alloantigen with similar intensity and precursor frequencies (Akbar et al 1988; Merkenschlager et al. 1988; Yamashita et al 1989; Takeuchi et al 1989). This project shows that these conclusions extend to minimal requirements in addition to optimal conditions. Elevated levels of second messengers, increased expression of accessory molecules and pre-formed signaling complexes which are detected in RO^+ T cells but not in RA^+ T cells have led many researchers to conclude that RO^+ T cells are more sensitive to activation than RA^+ T cells (Robinson et al. 1993; Dianzani et al. 1992; Sanders et al. 1988). However, this is an over simplification that ignores many of the stimuli listed in Table 8-I and is clearly not the case for allogeneic stimulation.

RA^+ T cells were thought to have more stringent activation requirements and a greater dependence on the presence of a co-stimulus (Larsen et al. 1992; Damle et al. 1992; Kuiper et al. 1994). RO^+ T cells are able to respond to CD3 and CD2 MAbs in the absence of a co-stimulus, RA^+ T cells cannot (Moore and Nesbitt, 1987; Ferrer et al. 1992; Kuiper et al. 1994). This evidence coupled with studies with soluble antigens drew many researchers to the conclusion that RA^+ T cells were naive cells and RO^+ T cells were memory T cells. However, data presented here shows firstly that neither CD45R population can respond to activation through the TCR unless a co-stimulus is present. This co-stimulus can be either CD54 or CD58, contrary to previous studies showing that RA^+ are preferentially utilize the LFA-1/ICAM-1 pathway (Kuiper et al. 1994). Secondly, RA^+ and RO^+ T cells respond equally to alloantigen presented either on a transfected cell line or naturally expressed on an LCL. Alloantigen responses do not comply with the naive/memory T cell model since memory (RO^+) T cells should only respond to an antigen that the host has previously encountered.

Table 8-I: Functional differences between $CD4^+RA^+$ and $CD4^+RO^+$ T cells *in vitro.*

Stimulus	$CD45RA^+$	$CD45RO^+$	Reference
Soluble antigen	-	+++	(Tedder et al. 1985; Smith et al. 1986; Merkenschlager et al. 1988; Beverley et al. 1992)
PWM	-	+++	(Morimoto et al. 1985; Ferrer et al. 1992)
superantigen	-	+++	(Horgan et al. 1990)
CD3 MAbs	+/-	+++	(Sanders et al. 1989 Welge et al. 1993; Patel et al. 1994)
CD2 MAbs	+	+++	(Sanders et al. 1989; Kanner and Ledbetter, 1992)
allogeneic MLR	+++	+++	(Akbar et al 1988; Merkenschlager et al. 1988; Yamashita et al 1989; Takeuchi et al 1989)
PMA+Io	+++	+++	(Wallace and Beverley, 1990)
Crosslinked CD3	+++	+++	(Schwinzer et al. 1992a; Schwinzer et al. 1992b)
sAg + APC	+++	+++	(Fischer et al. 1992)
PHA	+++	+	(Morimoto et al. 1985; Tedder et al. 1985; Wallace and Beverley, 1990)
ConA	+++	+	(Morimoto et al. 1985; Tedder et al. 1985; Sanders et al. 1988)
Autol. MLR	+++	+	(Akbar et al 1988; Takeuchi et al 1989)
TCR MAb	+++	+/-	(Schwinzer et al. 1992a; Schwinzer et al. 1992b)

With the exception of alloantigen and mitogens, stimuli involving the TCR/CD3 complex preferentially activate RO^+ T cells and have little effect on RA^+ T cells at low doses (Table 8-I). CD3 MAbs, soluble antigen, CD2 MAbs and superantigen all stimulate T cells by direct interaction with the CD3/TCR complex and preferentially activate RO^+ T cells. It was concluded from these studies and evidence outlined in Chapter 1 that RO^+ T cells selectively respond to activation through the TCR/CD3 complex. If RO^+ T cells were more susceptible to activation through the TCR compared to RA^+ T cells, how does this alteration occur? In B cells, primary proliferation is accompanied by a switch from low to high affinity B cell receptor expression (Vitetta et al. 1991).

Since the TCR does not undergo somatic mutation which would increase receptor affinity, alternative changes such as elevated expression of accessory molecules and pre-formed signaling complexes could increase susceptibility to activation through the TCR during the switch from RA^+ to RO^+. This would also enable RO^+ T cells to respond to lower concentrations of antigen *in vivo*. However, the CD3/TCR activation pathway is functional in RA^+ T cells since these cells are responsive CD3 MAbs, CD2 MAbs and superantigen at very high concentrations (Kuiper et al. 1994).

MAbs against the CD3 complex bypass the TCR-peptide-MHC interaction and directly activate the transmembrane signaling complex. However, a soluble CD3 MAb, OKT3, does not induce elevated levels of tyrosine phosphorylation or Ca^{2+} mobilization in either CD45R T cell population (Schwinzer et al. 1994). It does however, activate the src kinase $p21^{ras}$ in RA^+ but not RO^+ T cells, although the molecule is detectable in both populations (Schwinzer et al 1994). It can be concluded from this evidence that CD45R T cells have different transmembrane signaling pathways associated with the TCR. (Patel, 1994; Schwinzer, 1994). CD2 MAbs can also bind to RA^+ and RO^+ T cells but only activate RO^+ T cells. The reason for this distinction is either due to the level of CD2 expression on the cell membrane or the sensitivity of the activation pathway. Activation by this method induces phosphorylation of $p56^{lck}$ but levels of this src kinase are equal in CD45R populations (Rothstein, 1993). The expression of CD2 however, is lower in RA^+ T cells. implying that the number of CD2 molecules cross-linked by CD2 MAbs may be too low to induce activation in RA^+ T cells.

Investigations using limiting dilution assays found a high frequency of recall antigen responses in RO^+ T cells that were not detected in RA^+ T cells (Merkenschlager et al. 1988). However, both populations responded to alloantigen with similar frequency and intensity. Foreign antigen must be processed and presented to specific T cells by autologous APC to induce an immune response. T cell responses against soluble antigen are dependent on the peptide being taken up by a APC, processed and presented to T cells, expressing antigen specific TCR which are in continual circulation of the blood stream. For allogeneic responses, the antigen can be presented by allogeneic APC as well as processed by host APC.

This means that a higher proportion of APC are expressing alloantigen and increases the probability of an alloantigen specific T cell encountering and APC. This coupled with the fact that both populations of CD45R T cells are able to respond to alloantigen results in a more vigorous response in comparison to soluble antigen.

The number of MHC molecules on an APC containing alloantigen is also higher than for soluble antigens. Although autologous APC would process alloantigen as it would soluble antigen and is likely to result in similar numbers of autologous MHC molecules expressing a particular antigen, a higher proportion of MHC molecules on an allogeneic APC would contain alloantigen. The T cell-APC interaction would therefore involve a larger number of ligand interactions during cell-cell contact. This would result in the induction of a stronger stimulus through interactions which although weaker than those involved in soluble antigen presentation, induce a stronger signal in total. This would also create a greater number of potential transmembrane signals (through the TCR and co-stimuli). The net result would be a stronger activation signal which may not be as dependent on co-stimulation and would explain why both RA^+ and RO^+ T cells are able to respond. A similar type of activation is seen in naive B cell responses which involve numerous interaction between low affinity antibodies to induce activation (avidity verses affinity). In the case of RA^+ T cells, the number of interactions overcomes the reduced accessory molecule expression. In soluble antigen responses, the number of T cell-MHC molecule interactions are lower and maybe more dependent on co-stimulatory signals, giving RO^+ T cells a distinct advantage over RA^+ T cells.

Recent studies have revealed that both the number of TCR engagements and the affinity of the ligand for the TCR play a major role in determining the outcome of T cell stimulation. The threshold levels of activation as determined by the number of TCR engagements is approximately 8000, irrespective of the type of antigen and the affinity of the MHC molecule for the TCR. Although this has not been investigated in CD45R populations, it is tempting to speculate that soluble antigen stimulated cells involve a low number of high affinity TCR-MHC/peptide interactions and alloantigen would involve a high number of low affinity interactions (Viola et al 1996).

In summary, RA^+ and RO^+ T cells both have the potential to respond equally to antigen, it is the strength of the signal that plays a vital role. However *in vitro*, APCs are exposed to optimal concentrations of antigen yet RA^+ T cells fail to respond.

This does not mean that all MHC molecules on the APC will contain foreign antigen, it does mean that the number of potential interactions between the T cell and APC is increased.

The phorbel ester phorbel myristate acetate (PMA) in the presence of a calcium ionophore (Io), directly activates PKC and is a vital component of the transmembrane signaling pathway. PMA induces proliferation in RA^+ T cells and not RO^+ T cells (Budd et al. 1987; Wallace and Beverley, 1990). However, the PKC α, δ and γ are expressed equally in CD45R populations, implying that the difference lies further down the signaling pathway. Alternatively, this pathway could be regulated by a negative feedback mechanism preventing activation and maybe even inducing cell death in RO^+ T cells (Schlunck et al. 1990). The second explanation could also apply to mitogenic activation which also preferentially activates RA^+ T cells (Morimoto et al. 1985; Tedder et al. 1985). Autologous MLRs are also selective for RA^+ T cells (Morimoto et al. 1985), however, the mechanism for autologous antigen activation is poorly understood but involves MHC class II expressing cells.

The lack of response by RO^+ T cells may be due to the absence of cytokines necessary for proliferation such as IL-2. Although RO^+ T cells are able to produce low levels of this cytokine, the concentration may be too low to induce and sustain activation and therefore the cells apoptose. T cell triggering is thought to involve either the formation of complexes between CD4, CD3 and TCR or conformational changes in the TCR structure (Jameson and Bevan, 1995; Janeway, Jr. 1995). It is possible that both of these mechanisms are at work during T cell activation and the stimulus dictates which triggering mechanism is the initial signal or is more dominant. Since RO^+ T cells are believed to possess pre-formed signaling complexes giving this subset an advantage over RA^+ T cells, alloantigen must either involve conformational changes in the TCR to trigger T cell activation or have less stringent co-stimulatory requirements (see below). Presentation of a soluble antigen involves the association of the peptide, MHC and TCR. Normally, the specific interactions between these three components results in proliferation of RO^+ T cells. This may come about because the peptide forces a conformational change in the TCR which leads to signal transduction (Jameson and Bevan, 1995; Janeway, Jr. 1995). In alloantigen stimulation, the peptide and the MHC are foreign to the T cells and may induce more pronounced conformational changes in the TCR on a larger number of T cells.

Superantigens mimic soluble presentation by forcing contact between the TCR and the MHC complex by binding to the V regions of the TCR (Hurley et al. 1995). Such an action may also induce the necessary alterations in TCR topology to activate the transmembrane signaling pathways.

Priming of naive T cells results in a population of T cells that respond more vigorously and more rapidly to the same antigen on re-exposure. My studies showed that both populations of T cells were able to respond to alloantigen in a primary response. Priming of whole PBMC with HLA-DR alone was not sufficient to produce true alloantigen specific T cells. However, priming with a cell expressing allogeneic MHC and co-stimulatory signals led to the generation of primed cells able to respond to alloantigen in the absence of co-stimulatory signals when rechallenged. Although the involvement of murine accessory signals being involved cannot be ruled out, these cells are unable to aid in the priming of T cells to alloantigen indicating that their involvement in the activation of human T cells is minimal. This shows that secondary responses to alloantigen were not as dependent upon co-stimuli as the primary responses. Similar conclusions concerning co-stimulation dependence have been drawn from studies discussed earlier in this section using CD3 and CD2 MAb responses (Kuiper, 1994; Ferrer, 1992; Moore and Nesbitt, 1987).

Contrary to our predictions, RO^+ T cells cannot respond to alloantigen any more rapidly or vigorously when the minimal types of signals are provided. Our data does not comply with the general dogma stating that memory cells are the more sensitive to activation than naive T cells. Only with respect to cytokine requirements did RO^+ T cells demonstrate any clear difference over RA^+ T cells against alloantigen.

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Appendix 2-I:Expression of Activation Molecules on Purified $CD4^+$ CD45R Subsets

Antigen	$CD4^+CD45RA^+$	$CD4^+CD45RO^+$
TCRαβ	96 ± 2.9	97 ± 2
CD19	1.8 ± 0.3	1.9 ± 2.2
CD16	1 ± 0.2	4.1 ± 3.2
CD14	1 ±0.4	1.2 ± 0.5
CD45RA	94 ± 2.8	3.9 ± 2.9
CD45RO	8.5 ± 2.3	93.9 ±9.1
RA/RO	6.9 ± 1.5	7.6 ±6.6
CD4	95 .4 ± 5.5	96 ± 9.7
CD8	2 ± 1.2	2.6 ± 1.9
HLA-DR	4.8 ± 2.7	3.9 ± 1.8
CD54	10.8 ± 7.1	25.8 ± 19.4
CD58	20.0 ± 17.2	65.4 ±20.5
CD25a	6.1 ± 4.4	32 ± 19
CD69	6.9 ± 2.4	9.5 ± 2.9
CD26	40.2 ± 23.6	53 ± 15.4
CD38	46.4 ± 15	36.6 ± 17.5
CD11a/CD18	71.7 ± 15.1	76.6 ± 12.3
CD2	68.5 ± 14.1	83.7 ± 8.1
CD28	82.2 ± 3.4	97.4 ± 1.4
CD50	87.6 ± 2.2	95 ± 3.3
CD102	74.3 ± 3.9	85.2 ± 1.5
CD45RB	73.4 ± 4.7	95.9 ± 3.7

The percentage of positive cells expressed as mean ± SEM for specified antigen expression on 15 individual donors.

APPENDIX 2-II: LIST OF MATERIALS

Butterflies - 21 gauge (Abbot, Ireland)

Centrifuge, MSE Mistral 200 (Fisons, Loughborough, UK)

Centrifuge tubes 10ml & 50ml (Greiner Ltd, Germany)

CTLL, IL-2 dependent cell line (ECACC, Pooton Downs, UK)

Culture flasks, 25cm3 and 250cm^3 (Falcon, Oxford, UK)

Culture plates, flat-bottomed, 24 and 96 well (Falcon, Oxford, UK)

Di sodium EDTA diluted in distilled water (Sigma, Poole, UK)

Distilled water (Baxter Healthcare Ltd, Thetford, UK)

FACSCAN (Becton Dickenson, Oxford UK)

FCS heat inactivated for one hour @ 56°C (FCS; ICN, Thame, UK)

Gelatin, 2% in tissue culture grade water (Sigma, Poole, UK)

Geneticin (ICN, Thame, UK)

Glass beads, acid washed, 0.3mm diameter (Philip Harris Scientific, London, UK)

Glass wool (Philip Harris Scientific, London, UK)

Heparin tubes (10ml plastic tubes, Stardstedt, Germany)

Hoffmans Balanced Salt solution without sodium bicarbonate-10 times concentrated and diluted with sterile, distilled water (HBSS; ICN, Thame, UK)

Hoffmans buffer - 8.3mg/100mls Ammonium chloride, 73mg/100ml EDTA and 1g/100ml Potassium carbonate dissolved in 100mls of distilled water to make 10 times stock solution. (all chemicals from Sigma, Poole, UK)

Hypoxanthine (6-hydroxypurine) anhydrous powder diluted in distilled water (Sigma, Poole, UK)

Incubator -5% CO2 , 37°C, 100% humidity (ICN, Thame, UK)

Instat-2, statistical software, (Graphpad, San Diego, USA)

L-glutamine, 200mM (ICN, Thame, UK)

Liquid scintillant β counter (Wallac, Turku, Finland)

Liquid scintillant, high flash point LSC cocktail (Packard, Groningen, Netherlands)

Lymphoprep pH 6.8 (Nycomed, France)

Magnet - Biomag separator (Metachem diagnostics Ltd Northampton, UK)

Magnetic beads coated with goat anti mouse IgG or IgM,1μm, 1mg/ml in phosphate buffer pH 7.4 with EDTA and azide (Metachem diagnostics Ltd, Northampton, UK)

Mitomycin C (MMC; Kyowa Hakko Kogyo Co., Tokyo, Japan)

Mycophenolic acid (6-[4 hydroxy-6 methoxy-7 methyl-3-oxo-5 phthalanyl]-4 methyl 4 hexenoic acid), dessicated powder diluted in distilled water (Sigma, Poole, UK)

Mycoplasma removal angent 50μg/ml in sterile distilled water (ICN, High Wycombe UK)

Mycoplasma testing kit including Hoechst stain and phenol red free HBSS (ICN, Thame, UK)

Needles - 21 gauge, sterile, (Sherwood medical, Ireland)

Neubauer chamber (BDH Ltd, Dagenham, UK)

Paraformaldehyde, 1% solution (Sigma, Poole, UK)

Penicillin (103 IU/ml) / Streptomycin (10 μg/ml) (ICN, Thame, UK)

Phosphate buffered saline solution +/- azide, pH7.3 (Oxoid, Basingstoke, UK)

Phytohaemagglutinin (PHA, Wellcome, Beckenham, UK)

RPMI 1640 without L-glutamine (ICN, Thame UK)

Saline (0.9% solution diluted in sterile distilled water)

Sephadex G10 (Pharmacia, Uppsala, Sweden) swollen in distilled water.

SRBC (TCS Ltd, Boltolph, UK)

Standardised pipettes; Gilsons 20μl, 200μl and 1000μl (Anachem, Luton, UK)

Sterile trypsin EDTA (0.05% trypsin, 0.02% EDTA in salt solution), (ICN, Thame, UK)

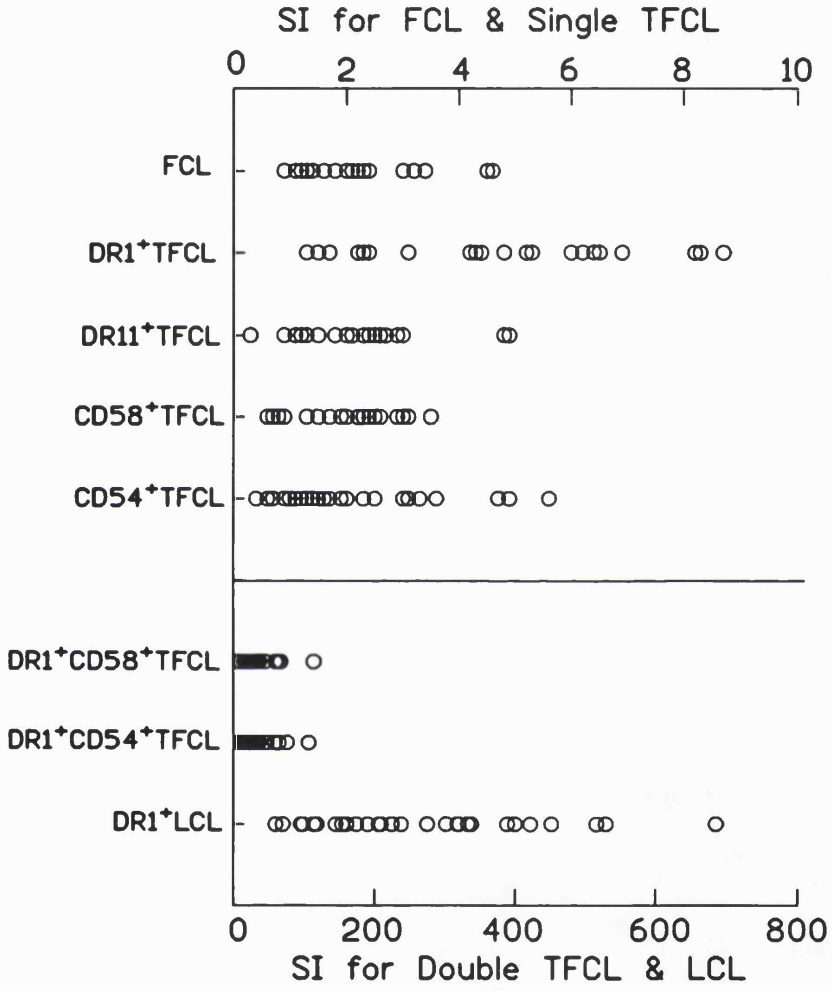
Sterile syringes, 10, 20 and 50ml (Monovette, Sherwood medical, UK)

Thymidine, [methyl-3H] 50 Ci/mmol specific activity at a concentration of 1mCi/ml in an aqueous solution (ICN Biomedicals, Thame, UK)

Trypan blue 1% in saline solution (Sigma, Poole, UK)

Xanthine (2-6 Dihydroxypurine) anhydrous powder diluted in distilled water (Sigma, Poole, UK)

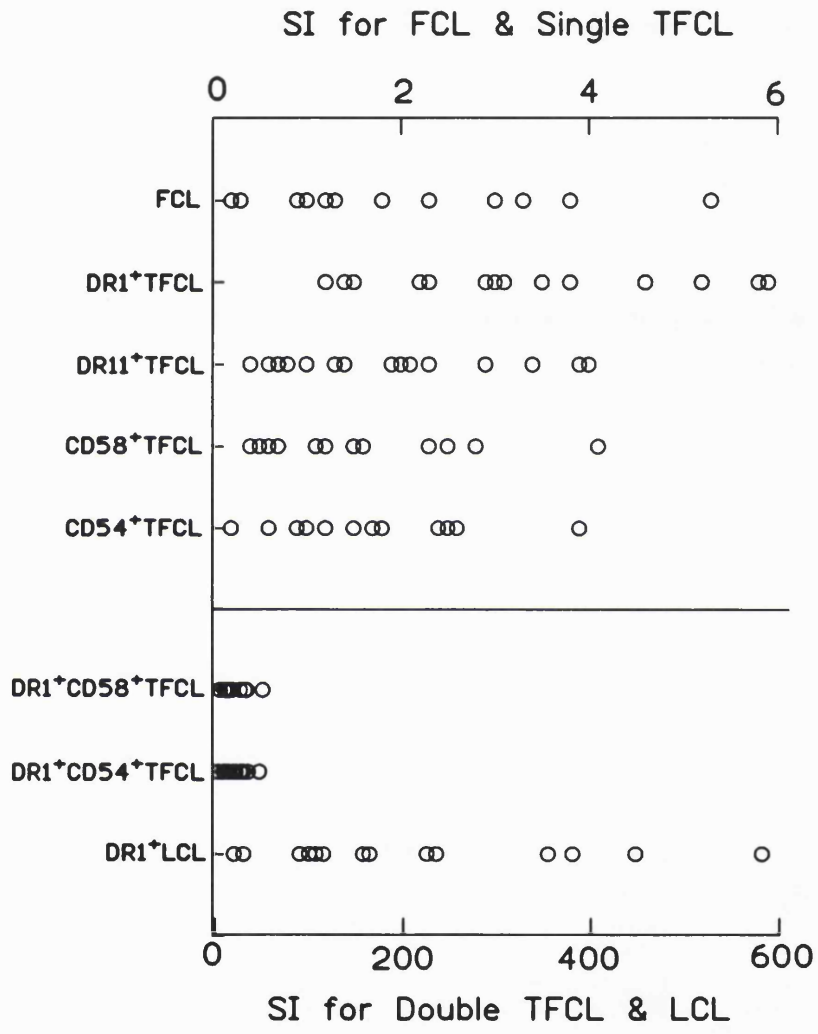
Appendix 3-I



Appendix 3-I: The response of PBMC to TFCL.

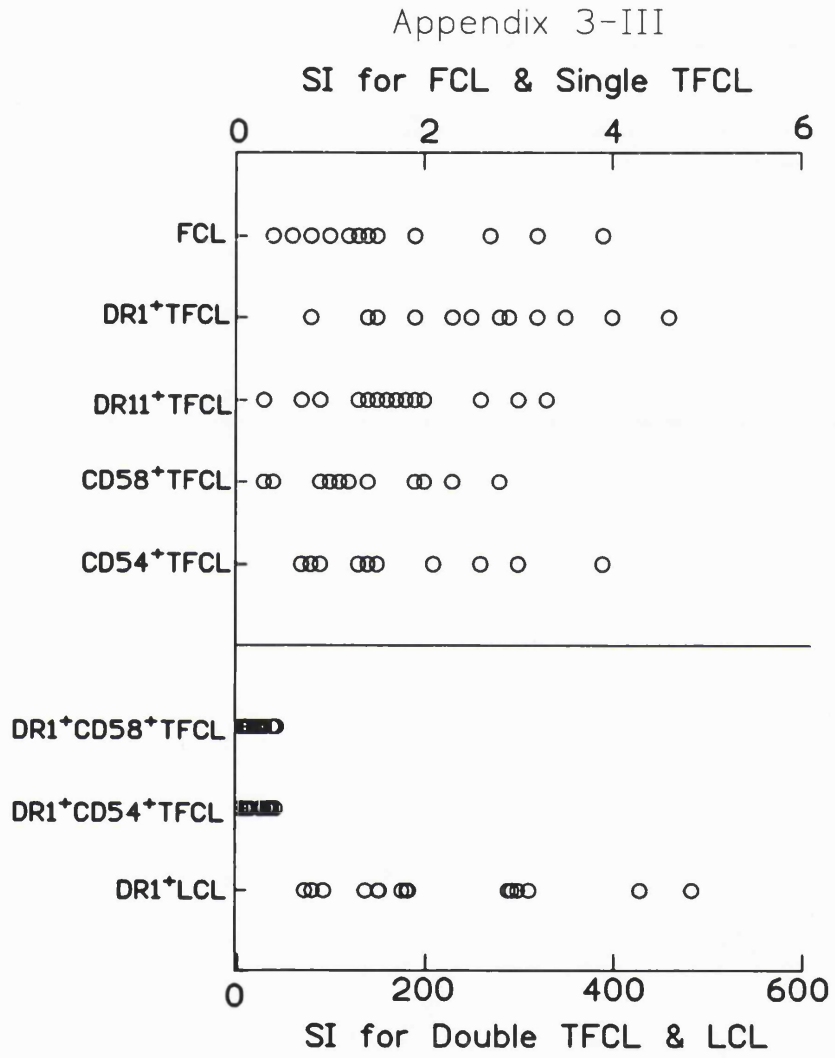
The responses of whole PBMC against single TFCL (top axis), double TFCL and $DR1^+$ LCL (bottom axis) by 30 individual HLA-DR1 negative donors. Each circle represents the SI of one individual and was calculated using resting responder cells as the control.

Appendix 3-II



Appendix 3-II: The response of CD45RA$^+$ T cells to TFCL.

The responses of whole CD45RA$^+$ against single TFCL (top axis), double TFCL and DR1$^+$ LCL (bottom axis) by 15 individual HLA-DR1 negative donors. Each circle represents the SI of one individual and was calculated using resting responder cells as the control.



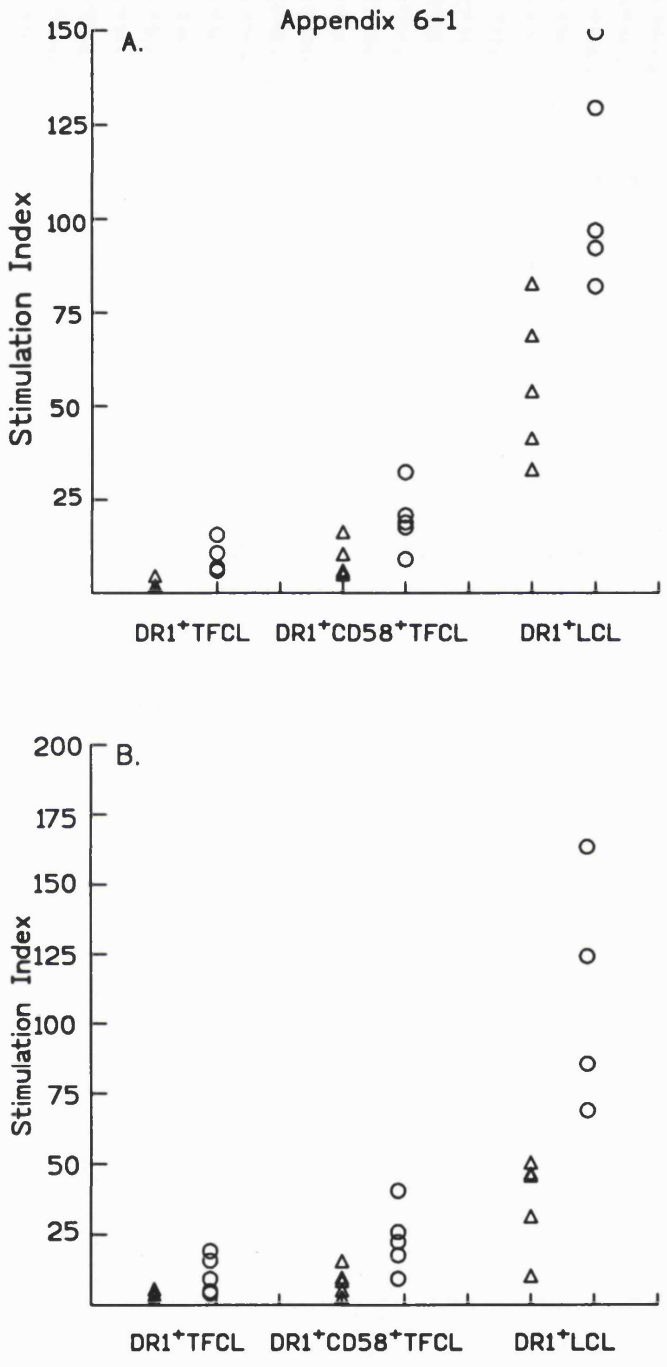
Appendix 3-III: The response of CD45RO$^+$ T cells to TFCL.

The responses of whole CD45RO$^+$ against single TFCL (top axis), double TFCL and DR1$^+$ LCL (bottom axis) by 15 individual HLA-DR1 negative donors. Each circle represents the SI of one individual and was calculated using resting responder cells as the control.

Appendix 3-IV: The response by PBMC, $CD45RA^+$ and $CD45RO^+$ T cells to TFCL.

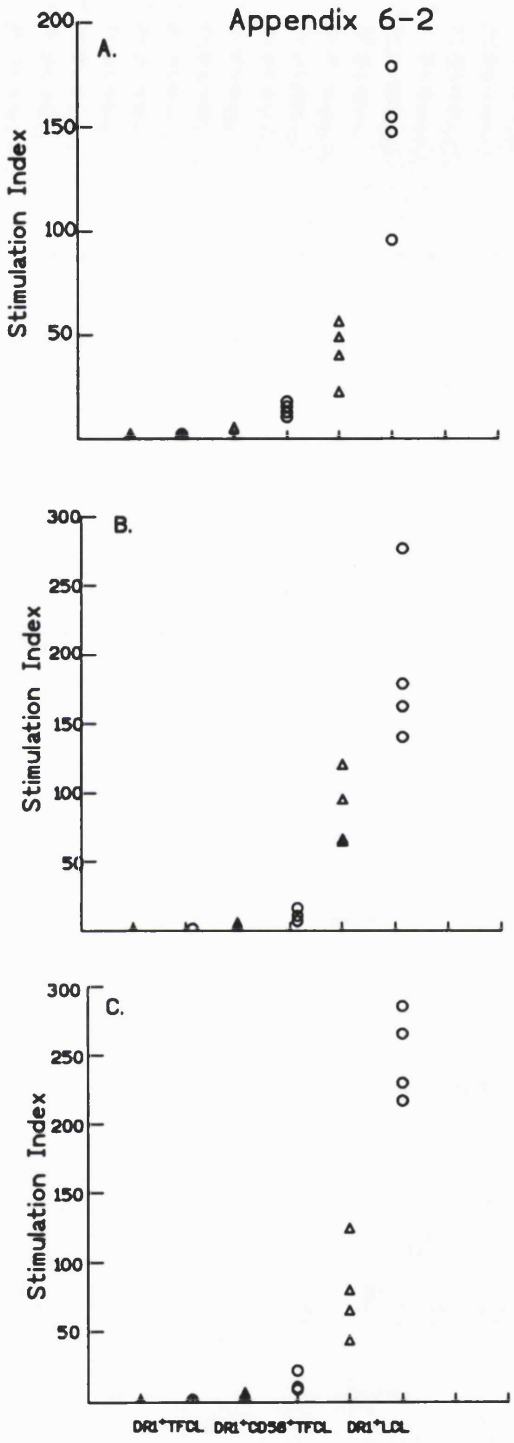
	PBMC	$CD45RA^+$	$CD45RO^+$
	(n=30)	(n=15)	(n=15)
FCL	2.21±0.96	2.27±1.76	1.59±0.93
$DR1^+$TFCL	4.23±2.21	6.18±3.67	3.57±1.01
$DR11^+$ TFCL	2.19±1.13	1.91±1.15	1.8±0.84
$CD58^+$TFCL	1.93±0.75	1.47±1.02	1.44±0.77
$CD54^+$TFCL	2.08±1.28	1.65±0.89	1.66±0.86
$DR1^+CD58^+$TFCL	32.59±22.03	22.54±12.06	23.63±12.28
$DR1^+CD54^+$TFCL	33.57±21.85	21.43±12.47	24.26±11.40
$DR1^+$ LCL	262.61±151.1	210±158.3	223.13±120.0

The mean ± SEM [^{3}H]TdR uptake of whole PBMC, $CD45RA^+$ and $CD45RO^+$ T cells stimulated with TFCL or $DR1^+$ LCL. Number represents cpm x 10^3 for HLA-DR1 negative healthy individuals.Mean and SEM were calculated using 30 separate samples of whole PBMC and 15 samples of CD45R populations (see numbers in brackets).



Appendix 6-I: The effect of autologous APC on alloantigen responses.

Individual responses of A.) $CD4^+CD45RA^+$ and B.) $CD4^+CD45RO^+$ T cell subsets from five HLA-DR1 negative individuals stimulated with $DR1^+$ TFCL, $DR1^+CD58^+$ TFCL and $DR1^+$ LCL in either the absence (open triangles) or presence (open circles) of autologous monocytes.



Appendix 6-II: Comparison of CD28 treatment on alloresponses by PBMC to $DR1^+$ LCL.

Freshly isolated A.) PBMC, B.) $CD4^+CD45RA^+$ and C.) $CD4^+CD45RO^+$ T cells from four normal healthy HLA-DR1 negative donors were stimulated with $DR1^+$ TFCL, $DR1^+CD58^+$ TFCL and $DR1^+$ LCL in either the absence (open triangles) or presence (open circles) of CD28 MAb.

Appendix 7-I-Activation molecules expressed on T cells stimulated with $DR1^+$LCL

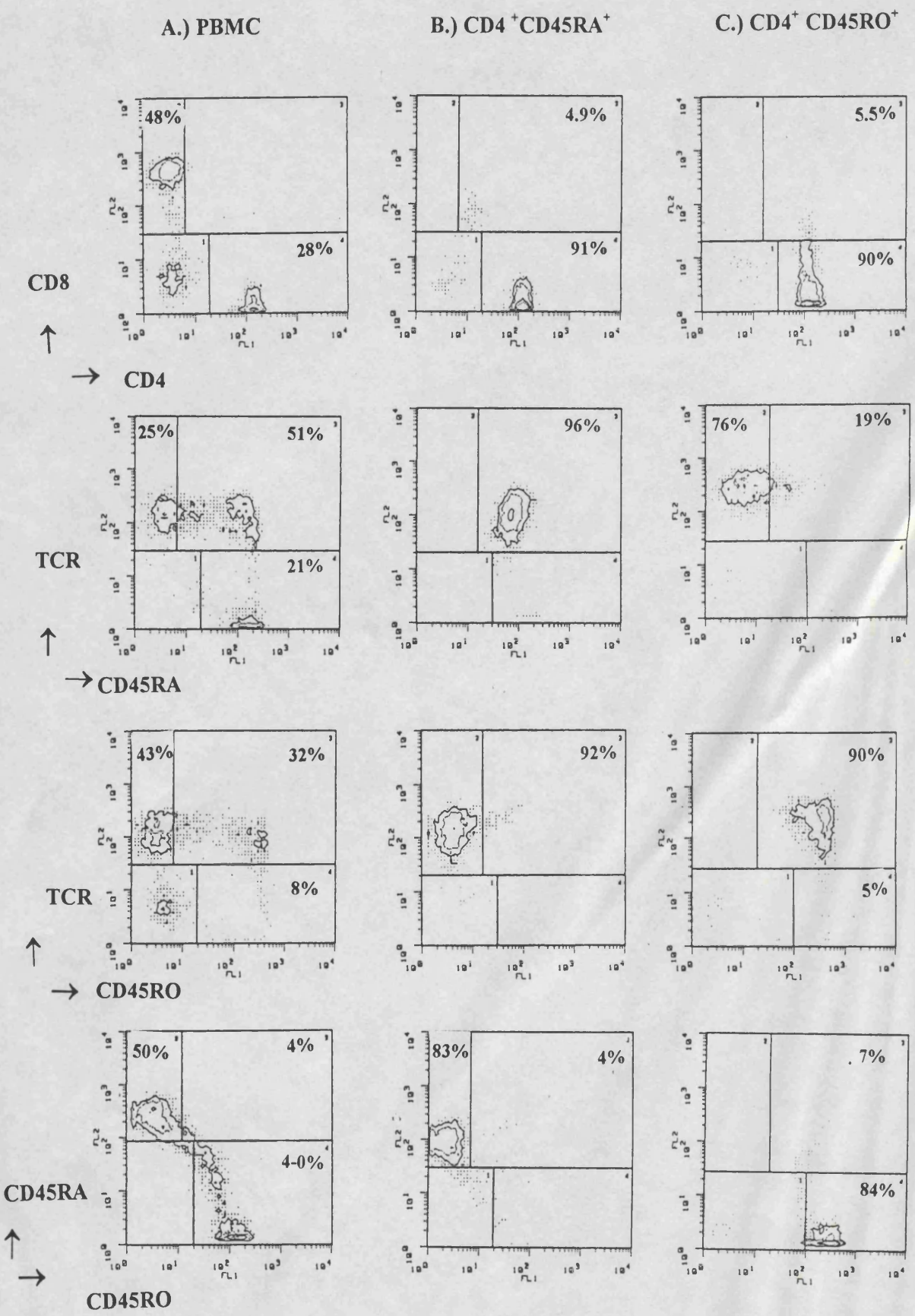
	Day 0	Day 1	Day 3	Day 5
TCR	68	72	78	67
MHC II	30	29	49	63
CD45RA	68	47	31	32
CD45RO	30	52	61	47
CD2	65	46	81	46
CD54	51	6	15	27
CD58	43	26	31	39
CD11a	71	76	79	43
CD25a	13	11	9	11
CD69	3	41	9	1
CD26	36	12	48	21
CD38	20	40	36	18
CD4	39	53	66	24
CD8	27	12	9	18
CD45RA/RO	51	6	13	23

Purified whole PBMCs stimulated in a primary MLR for five days with $DR1^+$ LCL. All cells described are gated on TCRαβ (with the exception of TCR itself). Numbers represent the percentage of T cells expressing the specified molecule.

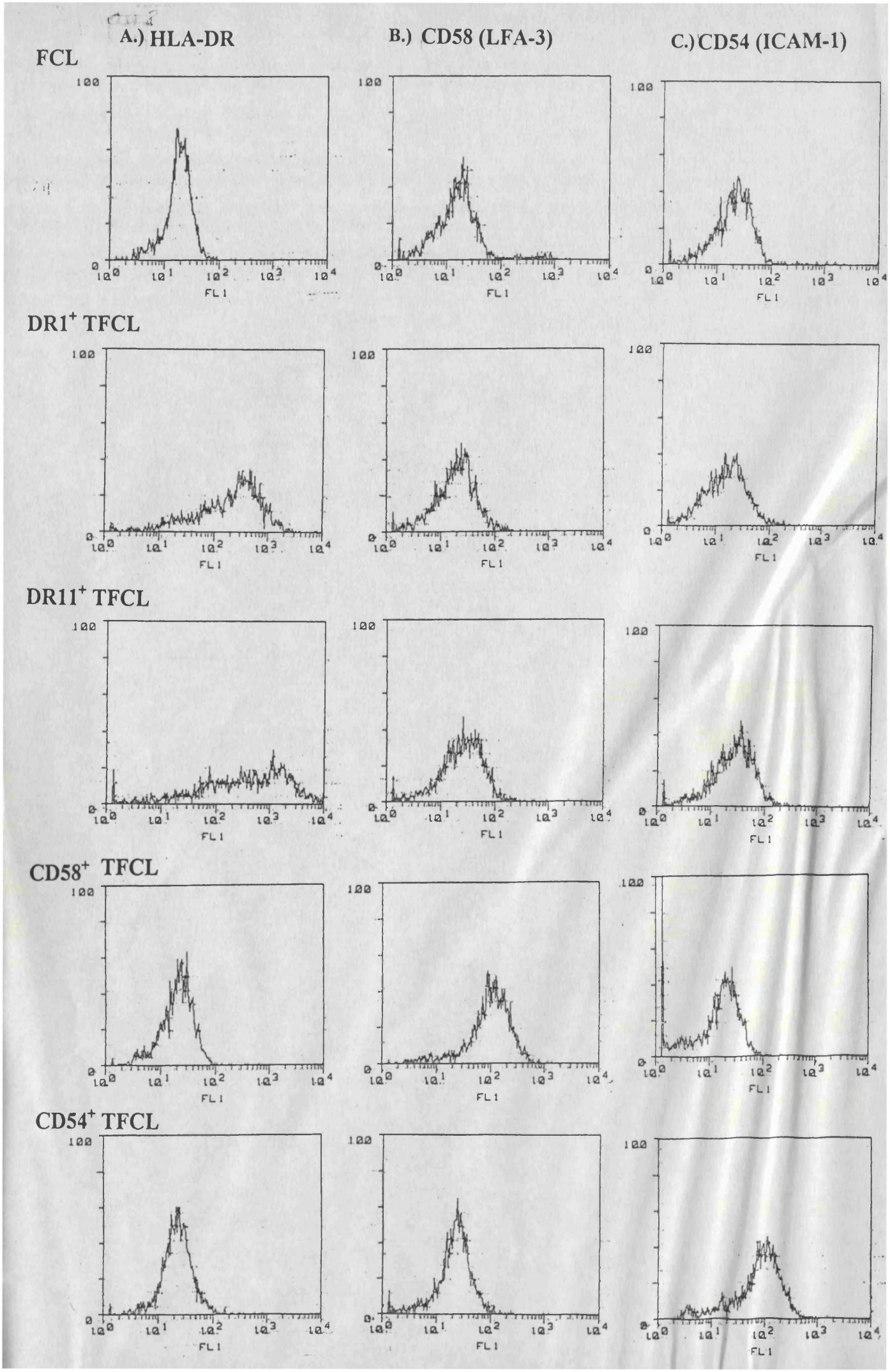
Appendix 7-II:-Activation molecules expressed on T cells stimulated with $DR1^+$TFCL

	Day 0	Day 1	Day 3	Day 5
TCR	68	77	83	79
MHC II	30	17	15	19
CD45RA	65	61	66	62
CD45RO	51	50	55	52
CD2	68	71	81	72
CD54	1	7	6	12
CD58	43	22	22	61
CD11a	71	73	81	65
CD25a	13	10	8	11
CD69	3	8	6	9
CD26	36	48	45	41
CD38	20	39	27	32
CD4	39	47	64	33
CD8	27	12	13	18
CD45RA/RO	51	7	8	39

Purified whole PBMCs stimulated in a primary MLR for five days with $DR1^+$ TFCL. All cells described are gated on TCRαβ (with the exception of TCR itself). Numbers represent the percentage of T cells expressing the specified molecule.

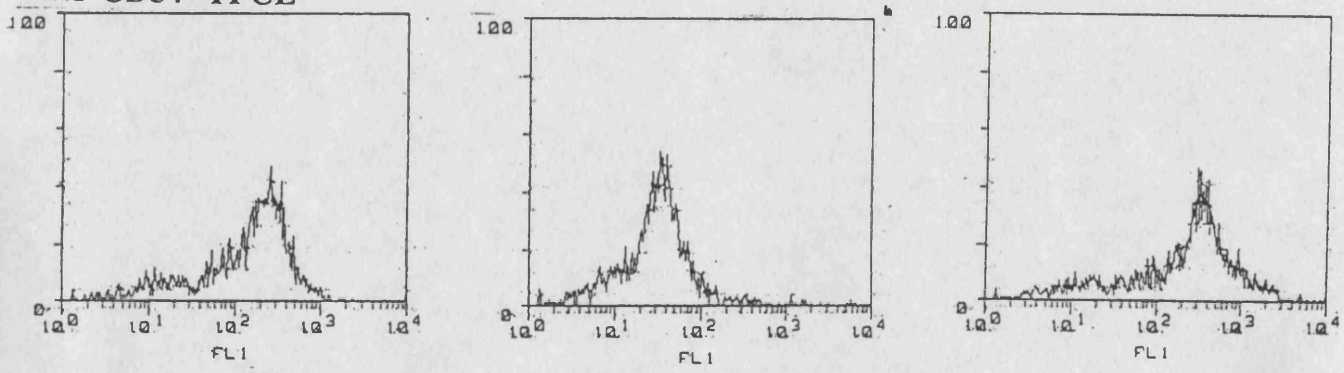


Appendix 2-IV: Facscan profiles of purified A.) PBMC; B.) CD4 $^{+}$CD45RA^{+} and C.) CD4^{+} CD45RO^{+} T cells. For details of staining method see Chapter 2.

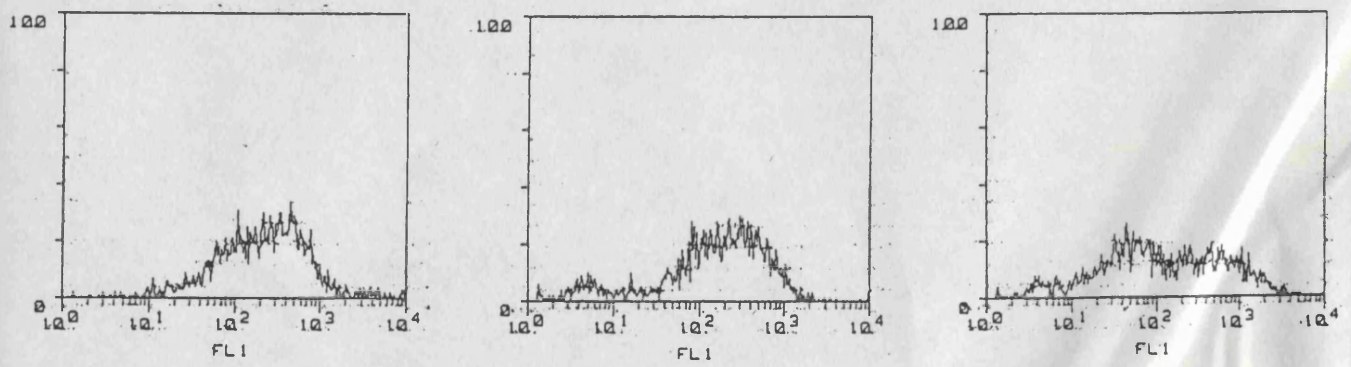

A.) HLA-DR
B.) CD58 (LFA-3)
C.) CD54 (ICAM-1)
FCL
DR1+ TFCL
DR11+ TFCL
CD58+ TFCL
CD54+ TFCL
100
0
FL1

HLA-DR CD58 (LFA-3) CD54 (ICAM-1)

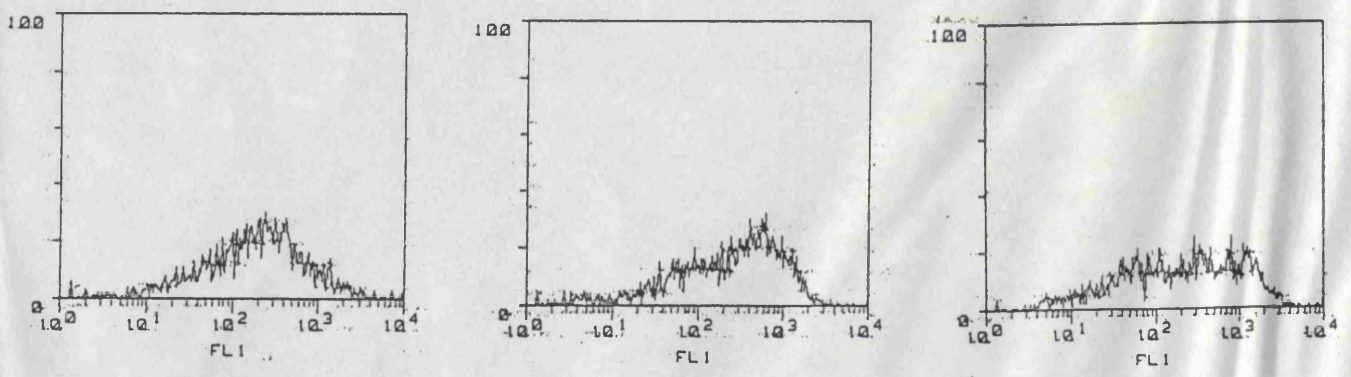
DR1^{+}CD54^{+} TFCL



DR1^{+}CD58^{+} TFCL



DR1^{+} LCL



Appendix 2-III: FACscan profiles of cell lines used as stimulator populations.