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Regulation of T Cell Growth.

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Abstract

This PhD thesis aims to examine the growth of T cells in response to cytokines of the common gamma chain (γc) family, in particular interleukin (IL)-2 and IL-15. Cell growth is commonly used to describe cells in exponential division however the growth of a cell is defined by its size and volume, which is directly related to the rate of cell metabolism and protein synthesis. Naïve T cells are small and circulate around the body maintaining a minimal rate of metabolic activity and protein synthesis. During an immune response naïve T cells undergo rapid proliferation and differentiate into effector cells. These cells produce and secrete large amounts of cytokines and effector molecules allowing them to mediate their immune function. The differentiation of antigen activated T cells to mature effectors takes several days and is regulated by cytokines. These cytokines need to maintain high rates of cell metabolism for a prolonged period. Thus the cytokines that regulate proliferation and differentiation of antigen activated T cells need to sustain cell growth.

Data presented in this thesis shows differential roles for gamma chain cytokines, specifically IL-2 and IL-15 in the regulation of protein synthesis and uptake of amino acids, whilst maintaining equal mitogenic capacity. This thesis highlights the possible uncoupling of the rate of cellular division and protein synthesis induced by cytokines and defines a unique role for common gamma chain cytokines in regulation of protein synthesis and ultimately the regulation of cellular function.

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Abbreviations

γc	Common gamma chain cytokine receptor (IL-2Rγ)
μ	Micro (10^{-6})
μM	Micro molar
μCi	Micro curie
μL	Micro litre
APC	Allophycocyanin
APC	Antigen Presenting Cell
ATP	Adenosine 5'-Triphosphate
BSA	Bovine Serum Albumin
Btk	Bruton's tyrosine kinase
CD	Cluster of Differentiation
CD25	IL-2Rα Chain
CD122	IL-2/15Rβ
CD127	IL-7Rα
$CD8^{IL-15}$	antigen activated CD8+ T cells cultured in IL-15
$CD8^{IL-2}$	antigen activated CD8+ T cells cultured in IL-2
CRAC	calcium release activated channels
cSMAC	Central Supramolecular Activation Cluster
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic lymphocyte associated molecule 4
DAG	Dyacyl Glycerol
DC	Dendritic Cell
DH	Dbl Homology
DMEM	Dulbecco's Modified Eagle's Medium
DN	Double Negative thymocyte ($CD4^-8^-$)
DNA	Deoxyribonucleic Acid
DP	Double Positive thymocyte ($CD4^+CD8^+$)
DTH	Delayed type hypersensitivity
ECM	Extra Cellular Matrix
EDTA	Ethylenediamine Tetra-Acetic Acid

ERK	Extracellular Signal-Regulated Kinase
ER	Endoplasmic reticulum
FACS	Flow Activated Cell Sorting
FBS	Foetal Bovine Serum
FITC	Fluorescent Isothyocyanate
FSC	Forward light Scatter
FITC	Fluorescent Isothyocyanate
FSC	Forward Scatter
FTOC	Foetal thymic organ culture
Gab2	GRB2-associated binding protein 2
GAP	GTPase activating protein
GDP	Guanosine Diphosphate
GEF	Guanosine exchange protein
Grb2	Growth Factor Receptor Bound Protein 2
GP	Glyco-Protein
GTP	Guanosine Triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulphonic Acid
HEV	High Endothelial Venule
hr(s)	hour(s)
^{3}H	Tritiated
HRP	Horse Radish Peroxidase
ICAM-1	Intracellular Adhesion Molecule-1
IFNγ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
IRS-1	Insulin receptor substrate 1
ITAM	Immuno-Receptor Tyrosine-Based Activation Motif
Itk	Inducible T cell kinase
Jak	Janus Activated Protein
JNK	Jun N-Terminal Kinase
JAM assay	Just Another Method
kDa	Kilo-dalton
L(l)	Litre
LAT	Linker For Activation of T Cells

LCMV	Lymphocytic Choriomeningitis Virus
LFA-1	Leukocyte Function Associated Antigen-1
LN	Lymph node
LT	Lymphotoxin
m	milli (10^{-3})
M	Molar
mA	Milli Amps
MAPK	Mitogen Activated Protein Kinase
ME	β-Mercaptoethanol
MEK	Mitogen Activated ERK Kinase
MHC	Major Histocompatibility Complex
MTOC	Microtubule organising centre
min	Minute
mL	Millilitre
Mr	Relative Molecular Weight
n	Nano (10^{-9})
NFκB	Nuclear Factor Kappa-B
NFAT	Nuclear Factor of Activated T Cells
ng	Nano (10^{-9}) gram
NK	Natural Killer (cell)
NK-T	Natural Killer T (cell)
nM	nano (10^{-9}) Molar
NP-40	Nonidet-P40
p38 MAPK	MAPK of 38 kDa
PBS	Phosphate-Buffered Saline
PdBu	Phorbol 12,13-Dibutyrate
PDK1	3-Phosphoinositide-Dependent Kinase 1
PE	Phycoerythrin
PH	Pleckstrin Homology
PI	Phosphatidyl Inositol (Phosphoinositide)
PI3K	Phosphatidyl-Inositol-3 Kinase (Phosphoinositide-3-Kinase)
PKB	Protein Kinase B
PKC	Protein Kinase C
PKD	Protein Kinase D

PLC	Phospholipase C
PMA	phorbol myristate acetate
Pre-TCR	Pre-T Cell Antigen Receptor
pSMAC	Peripheral Supramolecular Activation Cluster
PtdIns(3,4)P_2	Phosphatidyl-Inositol-3, 4-Diphosphate
PtdIns(3,4,5)P_3	Phosphatidyl-Inositol-3, 4,5-Triphosphate
PtdIns(4,5)P_2	Phosphatidyl-Inositol-4, 5-Bisphosphate
PTK	Protein Tyrosine Kinase
PVDF	Polyvinylidene Difluoride
-R	-Receptor (e.g. IL-2R)
r	Recombinant
RAG	Recombinase Activating Gene
RNA	Ribonucleic Acid
RT	Room Temperature
Ser	Serine
SCID	Severe Combined Immunodeficiency Disease
SDS	Sodium Dodecyl Sulphate
SH	Src Homology
Shc	Src Homology 2 containing protein
SHIP	Src Homology 2 [SH2]-containing Inositol 5-Phosphatase
SLC	Secondary Lymphoid Tissue Chemokine
SHP-2	SH2 domain-containing phosphatase 2
SLP76	SH2-Domain Containing Leukocyte Protein of 76 kDa
SMAC	Supramolecular Activation Cluster
SOS	Son of Sevenless
SP	Single Positive (either $CD4^+8^-$ or $CD4^-8^+$)
SRE	Serum Response Element
SRF	Serum Response Factor
SSC	Side Scatter
STAM	Signal-transducing adapter molecule
STAT	Signal Transducer and Activator of Transcription
TCR	T Cell (Antigen) Receptor
T_{CM}	Central Memory T cell
T_{EM}	Effector Memory T cell

TEC	Tec protein tyrosine kinase
Th	T Helper
Thr	Threonine
TNF	Tumour Necrosis Factor
Tris	Tris (Hydroxymethyl) Aminomethane
Tyr	Tyrosine
Vav	Vav oncogene
V.	Volume
V/V	Volume/Volume
V/W	Weight/Volume
VLA	Very Late Activation (Antigen)
Zap-70	Zeta (ζ) associated protein of 70KDa

Chapter 1

Introduction

Cells of the immune system can be subdivided into two different lineages, myeloid and lymphoid cells. These originate in the bone marrow and guard peripheral tissues by circulating around the body via the blood and the lymphatic system. The myeloid lineage includes monocytes/macrophages, dendritic cells (DCs), mast cells, granulocytes (polymorphonuclear leukocytes), neutrophils, basophils, and eosinophils. These cells are involved in immediate responses to infection and constitute the innate immune system which functions as the first line of defence against common bacterial and parasite infections. Lymphoid cells comprise two major cell types. B lymphocytes (B cells), which mature in the bone marrow and when activated differentiate into plasma cells that secrete antibodies and T lymphocytes (T cells) which mature in the thymus and control cellular immune responses. T and B cells mediate adaptive immune responses that provide a versatile system of defence acting to eliminate pathogens but also extending protection against subsequent re-infection with the same pathogen.

T cells function to recognise a unique antigen by means of a cell surface T cell receptor (TCR). The TCR is a multi subunit complex, which consists of two idiotypic disulphide bond linked polypeptide chains TCR α and TCR β in a complex with the invariant subunits of the CD3 antigen (CD3, ε, δ, γ and a

homodimer of ζ chains). There are two types of TCR polypeptide chains; TCR αβ expressed on T cells circulating in the peripheral blood and secondary lymphoid organs; TCR γδ expressed on T cells predominantly in the skin and gut.

TCR α, β, γ and δ subunits have both variable and constant regions and their structure is determined by somatic recombination of variable exons generating diversity. TCR α and β genes are composed of gene segments that encode variable (V), jointing (J) and diversity (D) regions. During thymocyte development, recombinase activating genes RAG-1 and RAG-2 drive random V (D) J recombination of these gene segments. The variability in recombination of the TCR α/β subunits generates diversity in the immune system and produces a population of T cells each expressing a receptor with unique specificity.

TCR ligands are not soluble antigenic peptides but peptides displayed by major histocompatability complexes (MHCs) on the surface of specialised antigen presenting cells such as dendritic cells (DCs). There are two main classes of T cells defined by the expression of CD4 and CD8 molecules, which act as co-receptors for MHC class II and MHC class I molecules, respectively. CD4+ T cells are referred to as T helper cells (Th) and are triggered by foreign antigens displayed in the context of MHC class II molecules to initiate a program of cytokine secretion that regulates the function of B cells and macrophages. CD8+ T cells recognise MHC class I bound antigenic peptides on the surface of cells infected with viruses or intracellular pathogens and can differentiate to effector killer cells that cytolytically destroy virally infected cells preventing the spread of an infection.

1.1 T cell development

T cell development takes place in the thymus and relays on an ordered sequence of differentiation and proliferation (**Fig 1.1.1**). T cell progenitors enter the thymus from the bone marrow proliferate and survive in response to the cytokines Stem Cell Factor (SCF) and interleukin 7 (IL-7). These early thymocyte progenitors lack expression of the major histocompatibility co-receptors CD4 and CD8 and are termed double negatives (CD4-CD8-, DNs). The commitment of these progenitors to the T cell lineage occurs when genes are rearranged at the loci encoding for components of the T cell receptor (TCR). Firstly, TCR-β locus rearrangements occur in the double negative compartment. If successful, the TCR-β chain is expressed on the surface and associates with a constitutively expressed pre-Tα chain. This complex associates with co-receptors of the CD3-complex, and is called the pre-TCR complex (1-4). The pre-TCR instructs cells to proliferate rapidly and express CD4 and CD8 co-receptors. CD4+CD8+ double positive (DP) thymocytes rearrange their TCR-α locus, express a mature TCR α/β complex and are subject to the processes of positive and negative selection that generates CD4+ and CD8+ single positive (SP) thymocytes. For a single T cell expressing a given TCR the outcome of positive or negative selection is dependent of the strength of the signalling response triggered by interactions between the TCR and MHC molecules loaded with self peptides (5, 6). Insufficient signalling i.e. a failure to recognise self MHC results in cell death by neglect; signalling in the optimal range allows positive selection; signalling exceeding the optimal threshold results in negative selection of thymocytes avoiding export of potentially self reactive cells into the periphery. These processes select T cells with moderate affinity for MHC-self-

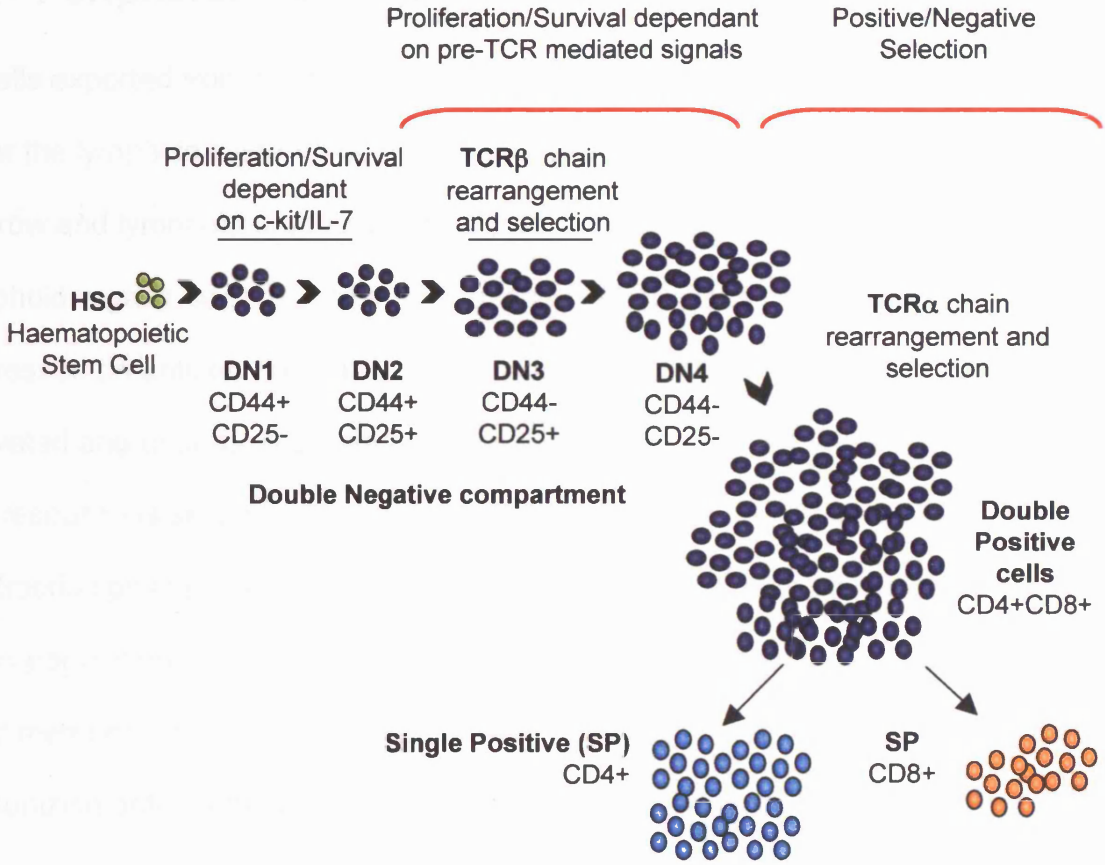


Figure 1.1.1: T cell thymocyte development. Hematopoietic stem cells migrate from the bone marrow to the thymus. These stem cells undergo a series of differential stages, defined by the expression of CD44 and CD25. This compartment lacks the expression of CD4+ and CD8+ co-receptors, double negative (DN). In the DN compartment rearrangement at the TCRβ locus occurs in the presence of a pre-Tα chain. If the pre-TCRαβ signals with an intermediate affinity for peptide presented by MHC molecules, present on thymic epithelium, the T cells progress to the double positive (DP) stage where CD4+ and CD8+ markers are up-regulated and T cells select for functional TCRα chains. DP go through positive and negative selection; low TCR affinity leads to death by neglect, strong binding leads to death, intermediate affinity results in survival and exit from the thymus.

peptide complexes but eliminate T cells with high or low-affinity for the complex (6).

1.2 Peripheral T cell differentiation

T cells exported from the thymus are resting or quiescent naïve T cells that enter the lymphoid system and re-circulate continually through blood, bone marrow and lymphoid organs. Upon infection naïve T cells in the peripheral lymphoid organs 'see' antigen in the context of MHC class I or II molecules expressed on antigen presenting cells, specifically dendritic cells (DCs), become activated and undergo a program of clonal expansion and differentiation. The T cell response is said to peak 5-7 days after infection and is followed by a contraction phase characterised by massive cell death of activated T cells. A small population of T cells can survive the contraction phase to persist as long-lived memory T cells that can respond very rapidly if re-exposed to the immunizing antigen thereby conferring protection against future re-infection.

1.2.1 CD4 T cells

When CD4+ T cells encounter cognate antigen they can differentiate into two distinct subsets both defined by their unique cytokine profiles (reviewed in (7)). Subsets, T helper1 (Th1) and T helper 2 (Th2) are responsible for cell-mediated inflammatory immunity and humoral i.e. B lymphocyte mediated responses, respectively. The hallmark cytokine of Th1 cells is IFN-γ, but they also produce IL-2, TNF-α and lymphotoxin (LT), known to mediate delayed type hypersensitivity (DTH) responses and macrophage activation. The signature cytokine of Th2 cells is interleukin 4 (IL-4), but these cells also secrete IL-5, IL-6,

IL-10 and IL-13, known to support B cell proliferation and differentiation and are critical for humoral-type immune responses (reviewed in (7)).

1.2.2 CD8 T cells

When CD8+ T cells encounter cognate antigen in the context of MHC class I and co-receptor stimulation they undergo a program of rapid cell cycle progression, increased cell size and growth. After the initial activation by antigenic peptide CD8+ T cells also undergo a program of differentiation and generate a pool of cytotoxic effector T cells (CTL) (for reviews see (8-10)). CTLs are programmed to lyse virally infected or tumorigenic cells using cytotoxic granules and Fas/Fas ligand mediated lysis. CTL carry an arsenal of lytic granules stored in specialised organelles in the cytoplasm. The controlled secretion of these lytic granules mediates the selective induction of target cell death (11-13). A key lytic granule protein is perforin that is responsible for the formation of 15-16nm pores in the membranes of target cells (14, 15). Pore formation is a key step in the induction of target cell death by facilitating delivery of lytic enzymes known as granzymes, which are serine proteases that trigger apoptosis (16-21). There are two types of granzyme: granzyme B that triggers apoptosis by caspase-cleavage (22) and granzyme A that initiates a caspase independent cell death by inducing nicks in DNA strands and preventing cellular repair (23, 24). Although a major route for the delivery of granzymes is via perforin induced pores, perforin-independent granzyme uptake has been demonstrated (25). For example, granzymes A and B can be internalised by endocytosis after binding the mannose-6-phosphate receptor (MPR) (26).

CTLs are capable of killing multiple targets in succession without harm to themselves (27). It is thought cytotoxic T cells protect against lytic granule damage by several mechanisms: 1. The expression of membrane bound cathepsin B, thought to cleave perforin on granule release preventing pore formation in the CTL membrane (28). 2. Expression of proteinase inhibitors called serpins thought to protect against granzyme B mediated killing (29). 3. Formation of the secretory synapse between target and killer T cell, thought to be the major determinant in maintaining CTL integrity during the killing (10, 30). This synapse formation is distinct from the immunological synapse known to occur between naïve T cells and APCs in the initial stages of an immune response. The secretory synapse of cytotoxic T cells is only formed for short periods of time on contact with target cells (minutes), where naïve T cell: APC interactions can last for hours (31, 32). Movement of secretory lysosomes containing cytotoxic granules is key to secretory synapse function. These lysosomes are known to move along microtubules towards and away from the microtubule organising centre (MTOC) where target cell recognition rapidly polarizes the MTOC towards the immunological synapse at the membrane (33-35). Small concentrations of lytic granules are secreted in the proximal region of synaptic contact, adjacent to the area where the TCR and co-receptors are clustered (31, 35, 36) and that the MTOC rapidly polarizes from one target to the next (37). Hence these synapses are made and destroyed very rapidly, facilitating an efficient killing process.

1.2.3 T cell Memory

The generation of memory T cells is critical for the development of protective immunity against repeat encounters with pathogens. Memory T cells are identified as small resting cells that become reactivated by cognate antigen which mediates the rapid expansion and production of effector cytokines at low activation thresholds. A large proportion of work has focused on CD8+ memory T cell generation and maintenance (reviewed by (9, 38-40)).

Two different populations of memory T cells have been described 'central memory' (T_{CM}) and 'effector memory' (T_{EM}) which differ in their tissue distribution and cytokine secretion profiles (41, 42). One hypothesis for the generation of T cell memory, proposes that T_{EM} and T_{CM} cells exist as stages of a dynamic process of maturation that takes place after the clearance of infection, in an antigen free environment (43, 44). There seems to be two different models of CD8+ T cell memory generation currently being debated; one suggests that CD8+ T cell memory occurs via a linear process of differentiation whereby memory T cells are thought to be direct descendents from effector T cells, i.e. Memory T cells are effector CTLs that for some reason do not die during the contraction phase of the immune response and persist in the lymphoid system for extended periods of time (43, 45-47). Alternative to the linear proposal is a model whereby the generation of memory T cells does not require that naïve T cells pass through an intermediate effector stage (48-52).

1.2.4 T cell Activation

Activation of T cells is initiated on engagement of the T cell antigen receptor (TCR) by antigenic peptides presented in the context of MHCs expressed on the

surface of APCs such as DCs. Signals produced by a number of co-stimulatory or accessory molecules such as CD28 and integrins such as LFA-1 co-stimulate T cells and are critical for T cell activation (53). Within seconds of encountering cognate antigenic peptide/MHC complexes on the surface of APCs T cells form a tight contact with the APC via the immunological synapse (32, 54-56). The immunological synapse is a highly ordered structure that is characterised by the segregation of receptors and signalling molecules into distinct concentric zones known as supramolecular activation clusters or SMACs. In particular, integrins and adhesion molecules are segregated into a peripheral zone or pSMAC, whereas the T cell antigen receptor accumulates into a central zone or cSMAC (56-59). The formation of the immune synapse is accompanied by the generation of a complex array of signal transduction pathways mediated not only by the TCR but also by other receptors in the contact zone notably the co-receptors CD4 or CD8 that mediate binding to MHC class II and I respectively. APCs also express co-stimulatory ligands, such as CD80 and CD86, which bind to the co-stimulatory receptors CD28 and CTLA-4 and play a key role in T cell activation. Adhesion molecules and integrins are also important regulators of T cell activation at this stage (56, 60-62). Signalling pathways initiated by antigen receptors and co-stimulatory molecules control transcription events that induce expression of cytokines and their receptors, which mediate T cell proliferation and differentiation culminating in lymphocyte activation or blastogenesis, central to adaptive immune responses.

1.3 T cell receptor (TCR) signal transduction

The TCR consists of idiotypic disulphide bond linked polypeptide chains associated with the CD3 antigen (CD3, ε,δ,γ) and a homodimer of ζ chains (**Fig 1.3.1**). When antigenic peptide/MHC complexes trigger the TCR, signalling is initiated by the invariant CD3 and ζ subunits of the TCR complex. These receptor-associated chains contain signalling motifs in their cytoplasmic tails termed immune receptor tyrosine-based activation motifs (ITAM). The CD3 γ, δ, ε subunits each have one ITAM and the ζ chain contains three. ITAMs are phosphorylated by Src protein tyrosine kinases (PTKs), specifically Lck. This leads to the SH2 (Src homology 2) domain-mediated recruitment of ZAP-70 (ζ-associated protein, 70 kDa), which phosphorylates key adapters, like the transmembrane adapter protein LAT (linker for activation of T cells) and SLP76 (SH2-domain containing leukocyte protein of 76 kDa).

One crucial protein that is recruited to LAT on TCR stimulation is phospholipase Cγ1 (PLCγ1). The activation of PLC results in the production of second messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate [Ins(1,4,5)P_3] (IP_3) by cleaving phosphatydylinositol 4,5 bisphosphate [PtdIns(1,4,5)P_2] at the plasma membrane. These second messengers are essential for T cell activation. DAG activates a number of proteins including serine kinases of the protein kinase C (PKC), PKD family and Ras guanyl-nucleotide-releasing protein (RasGRP) (63). Inositol 1,4,5 triphosphate (Ins(1,4,5)P_3 or IP_3) binds to IP_3 receptors on the surface of the endoplasmic reticulum (ER) and triggers the release of calcium stores into the cytoplasm from the ER. This event then triggers the opening of calcium-release-activated Ca^{2+} (CRAC) channels at the plasma membrane, allowing the influx of extra cellular Ca^{2+}. Elevation of

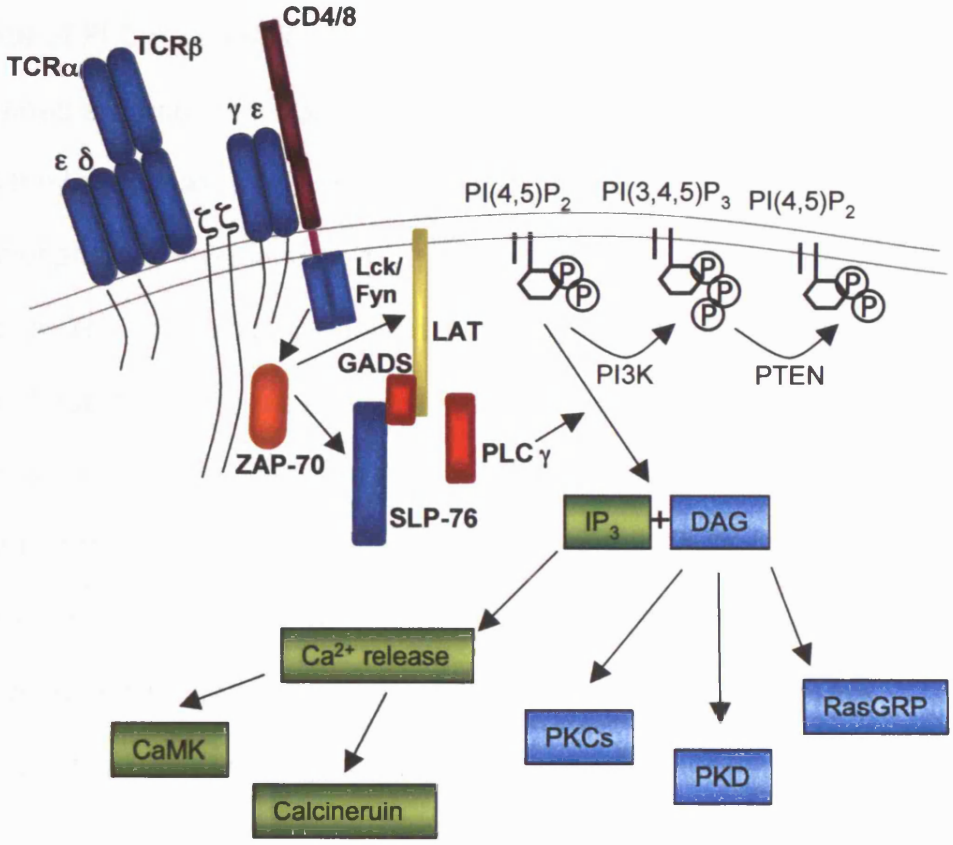


Figure1.3.1: Immediate T cell receptor signal transduction.
When the T cell receptor complex is engaged antigen receptor-coupled tyrosine kinases Lck/Fyn and Zap-70 phosphorylate down stream adaptors that trigger IP_3 and diacylglycerol (DAG) production via phospholipase C (PLC) mediated hydrolysis of PtdIns(4,5)P2. DAG binds to down stream effector molecules via a conserved C1 domain including various isoforms of PKC, PKD and Ras-GRP (activators of the GTPase Ras). IP_3 binds receptors on endoplasmic reticulum triggering the release of calcium. Intracellular calcium activates calcium/calmodulin dependent kinase (CaMK) and phosphatase calcineruin.

intracellular calcium activates the calcium/calmodulin dependent phosphatase calcineruin (64) and also results in activation of members of the calcium/calmodulin-depedent protein kinase (CaMK) family including CaMK type II (65-67), and CaMK type IV (68-71).

The importance of PLCγ1 regulation for lymphocyte activation stems from the fact that sustained elevation of intracellular calcium concentration ([Ca2+] i) is critical during the initial phases of T cell activation. In particular the regulation of the calcium phosphatase calcineurin, which is required for induction of cytokine gene expression. The importance of sustained calcium signalling in T cells is well established not least because a target for the powerful action of the immunosuppressive drugs cyclosporine A and FK506 is the calcium regulated serine/threonine phosphatase calcineurin. The important targets for calcium/calcineurin in lymphocytes are the NFAT (Nuclear Factor of Activated T cells) transcription factors that control antigen receptor induction of cytokine genes including the genes encoding IL-2, IL-4, GM-CSF, TNFα (72).

Other important signalling transduction pathway in T cells is mediated by the GTPase Ras (73). The guanine nucleotide binding cycle of Ras is controlled by guanine nucleotide exchange proteins (GEFs), which promote the transition from the inactive GDP-bound state to the active GTP-bound conformation, and GTPase activating proteins (GAPs) which stimulate the intrinsic GTPase activity of Ras resulting in hydrolysis of bound GTP to GDP. The nucleotide exchange reaction switches Ras on and the hydrolysis of GTP turns it off. p21ras rapidly accumulates in its active, GTP-bound form in antigen receptor activated T cells. Activation of Ras in T cells occurs through the recruitment of its exchange factor

Sos or RasGRP to the membrane. RasGRP contains a DAG binding C1 domain essential for its function. GTP-bound Ras leads to the activation of a number of serine/threonine kinases and dual-specificity kinases that are responsible for the eventual activation of the mitogen-activated protein (MAP) kinases Erk1/2.
One other key GTPase in T cell activation is the Rho family GTPase Rac (74). The involvement of Rac in T cell activation was initially suggested from biochemical studies where it was seen that the Rac GEF Vav-1 was tyrosine phosphorylated in response to antigen receptor ligation or engagement of the co-stimulatory molecule CD28 with its physiological ligands (75) Vav1 is a 95kDa protein with src homology (SH) 2 and SH3 domains and a Dbl homology region, which is a characteristic marker of GEFs for Rho family GTPases. T lymphocytes lacking expression of Vav-1 have global defects in TCR signal transduction including a failure to activate PLCγ signalling (76, 77). These defects are thought to arise from defects in cytoskeletal organisation and defects in cell adhesion.

TCR triggering also stimulates the production of the inositol lipid PI(3,4,5)P_3 which is produced when phosphatidylinositol-3 kinases (PI3K) phosphorylates PI(4,5)P_2 on the D3 position of the inositol ring (78, 79). Antigen receptors are thought to stimulate the activity of a PI3K complex that comprises a heterodimer of a regulatory p85 and a catalytic p110 subunit. Four isoforms of the p110 subunit have been described (α, β, γ, δ) and there are three mammalian genes that encode adapter subunits, p85α, p85β and p55γ (80). T cells express p110γ and p110δ and both these catalytic subunits appear to participate in lymphocyte signalling responses. p110δ is expressed primarily in cells of the immune

system and the defects in T and B cell activation in p110 δ deficient mice are consistent with a role for this particular PI3K isoform in antigen receptor signal transduction (81).

Models for PI3K activation invoke p85 binding to adapters which recruit the enzyme to the plasma membrane; constitutive membrane targeting of p110 catalytic subunits of PI3K creates a constitutively active enzyme that generates $PI(3,4,5)P_3$ and $PI(3,4)P_2$ when expressed in cells (82, 83). The candidate adapters for recruiting PI3K to the membrane in TCR activated cells are LAT or TRIM (TCR-interacting molecule) (84). The downstream targets of $PI(3,4,5)P_3$ and the signalling pathways regulated via PI3Ks will be discussed below (see section 1.5.4).

1.4 Gamma chain cytokines and their role in the immune system

Common gamma (γc) cytokines have been shown to play a central role in the development and differentiation of T cells (**Fig 1.4.1**). The crucial role of these cytokines in the immune system is illustrated by the development of X-linked severe combined immunodeficiency (X-SCID), in mice or humans with mutations in γc (85, 86).

1.4.1 X-Linked SCID

Severe combined immunodeficiency diseases (SCIDs) represent a spectrum of illnesses with similar clinical manifestations (87, 88)). Affected individuals have defects in both T and B cell compartments and without treatment die of

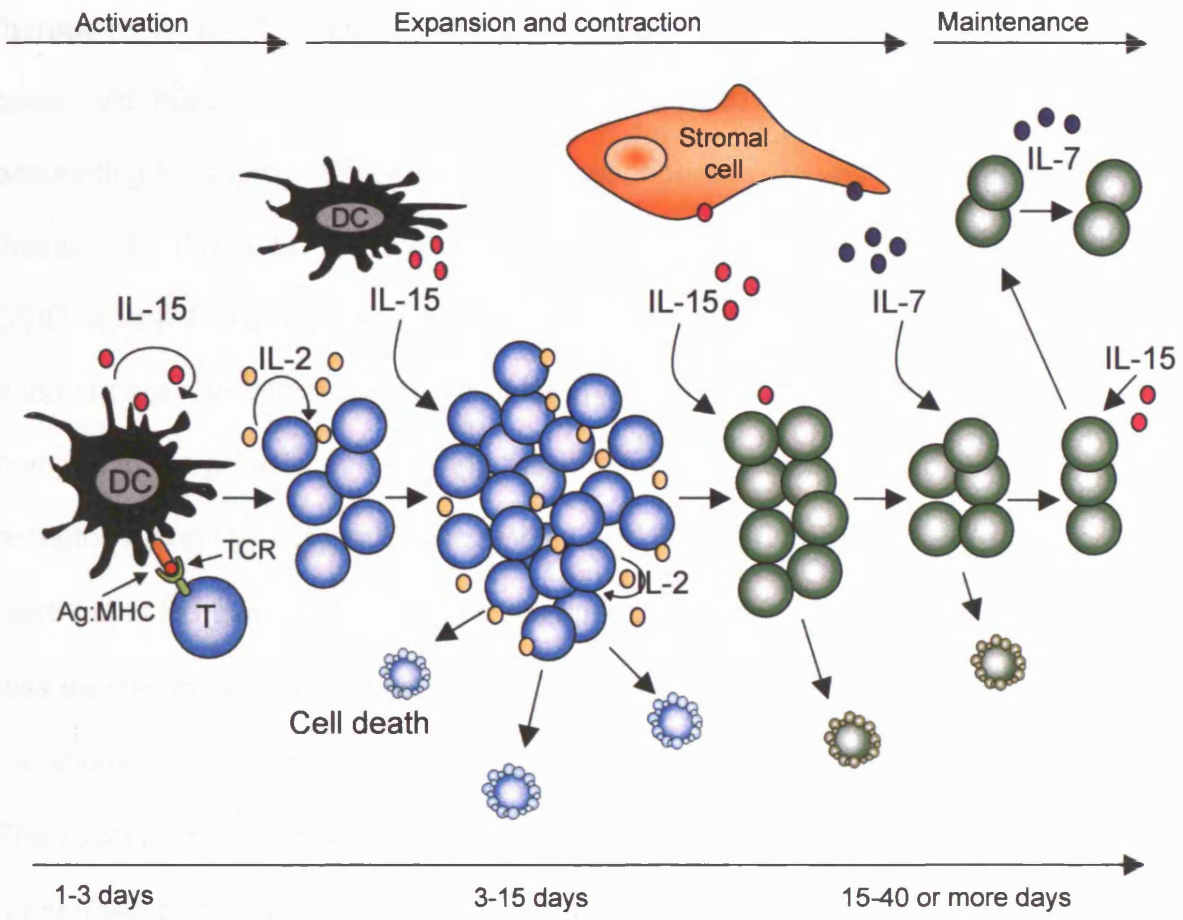


Figure1.4.1: Cytokines play a role in T cell differentiation *in vitro* and *in vivo*. T cell responses are initiated in the presence of pathogen by peptide:MHC complexes on dendritic cells (DC) interacting with T cell receptors (TCR). Activated T cells start to rapidly expand as they begin to secrete IL-2 and up regulate the IL-2 receptor (IL-2Rα). Activated T cells can exit lymphoid organs and migrate to sites of infection but eventually die resulting in the contraction phase of the response. Cytokines thought to influence T cell clonal expansion and differentiation *in vivo* and *in vitro* include IL-2, IL-15 and IL-7. IL-2 is a potent T cell mitogen and growth factor secreted by activated T cells. However, it is not clear if IL-2 solely drives T cell expansion *in vivo* as it does *in vitro*. IL-15 and IL-7 are secreted by bone marrow stromal cells. Cytokine gradients are thought to support T cell proliferation, differentiation and survival (IL-2 promotes cytotoxic T cell differentiation; IL-15/IL-7 the generation and survival of long lived memory T cells).

opportunistic infection within one year of life. SCID can be cured with a bone marrow transplant in most cases (89). Additionally there is active work on gene therapy for X-linked SCID, the most common form, although safety issues have arisen with this approach ((90-92). X-SCID is the most common form of SCID, accounting for approximately half of all cases, and is known as the bubble boy disease (87, 88). X-SCID is the main form of T, B and natural killer (NK) cell SCID, where T and NK cells are absent or diminished in number and B cell numbers are normal but function is ablated. The genetic cause of X-SCID was mapped to the gene locus encoding the common gamma chain cytokine receptor subunit (γc) of the IL-2R complex (93, 94). Moreover, X-SCID patients were found to have mutated γc subunits and experiments where the γc subunit was deleted in mice confirmed that the X-SCID phenotype results from mutations in the common cytokine receptor γ chain (95-97).

The γc was first identified as a subunit of the IL-2 receptor but it was quickly recognised that mice and humans deficient for IL-2 had a less severe phenotype when compared to that caused by γc mutation. Thus it was proposed that the IL-2 receptor γ chain was a component of additional cytokine receptors. Subsequently this receptor subunit (now named the common cytokine γ chain) has been shown to be a component of many cytokine receptors including; IL-4 (98, 99), IL-7 (100) (101), IL-9 (102, 103), IL-15 (104) and IL-21 (105). The function of these cytokines has been shown to be very important for T and B cell compartments, as well as mast and NK cell functions (**Fig 1.4.2**).

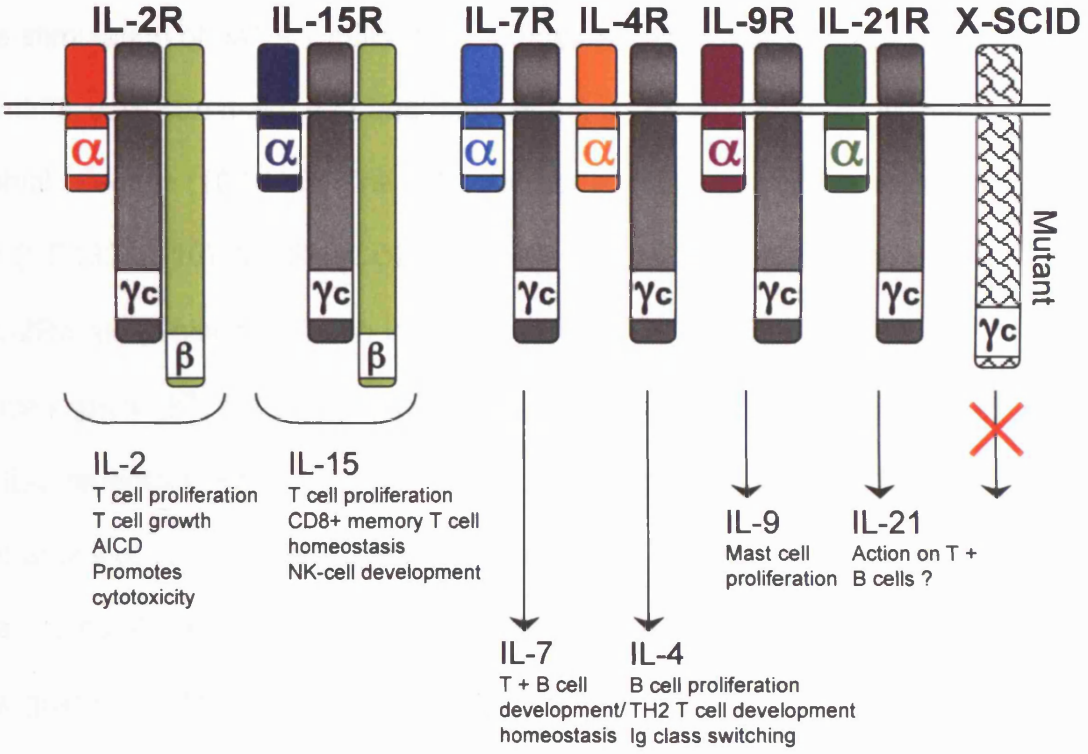


Figure 1.4.2: Actions of common γ chain family of cytokine receptors. γ_c is a component of the receptors (R) for interleukin (IL)-2, IL-15, IL-7, IL-4, IL-9 and IL-21. γ_c can exist independently of other chains and is recruited into cytokine receptor complexes by cytokine binding to high affinity unique α chains, or unique α and common β chain in the case of IL-2 and IL-15. Known functions of these cytokines are shown above where mutation of the common cytokine receptor γ_c results in X-linked severe combined immunodeficiency disease (X-SCID). *AICD; activation induced cell death, NK; natural killer, TH2 T helper 2, Ig; immunoglobulin.*

1.4.2 IL-2

Interleukin 2 (IL-2) is a four-bundle alpha-helical cytokine, which exerts its biological activity by binding the high affinity IL-2 receptor (IL-2R). The production of IL-2 and expression of the IL-2 receptor is initially triggered by immune stimulation of naïve T cells (106). It has also been described recently that dendritic cells can make IL-2 and express the IL-2 receptor when primed by a microbial infection (107-109). The IL-2 receptor is made up of three subunits, IL-2Rγc (CD132) (110), IL-2Rα (CD25) (111-114) and IL-2Rβ (CD122) (115-118). IL-2Rα alone binds IL-2 with low affinity (Kd = 10^{-8} M) but cannot transduce signals (87, 119, 120). The IL-2Rβ and γc together form intermediate affinity IL-2 receptors (Kd = 10^{-9} M) and can signal in the presence of high concentrations of IL-2. The high affinity IL-2 receptors (Kd = 10^{-11} M) comprise of all three chains, IL-2Rα/ IL-2Rβ/ IL-2Rγc, which can aggregate in response to very low gradients of IL-2.

Interleukin 2 (IL-2) was discovered in the late 1970's as the first T cell mitogen and was one of the first cytokines whose gene was cloned and sequences (121-123). IL-2 is an extremely potent mitogen *in vitro* and can be used to clonally expand antigen primed T cells. Indeed the discovery of IL-2 made it possible to produce large quantities of T cell clones *in vitro* and opened up many new avenues of research.

In T cells, expression of high affinity IL-2 receptors is not constitutive but is restricted to activated cells and is transient once T cells are deprived of immune stimulation (106, 124). *In vitro* activated T cell entry into cell cycle requires a critical threshold of IL-2/IL-2 receptor interactions and is thus limited by IL-2

concentration, cellular IL-2 receptor density and the length of exposure to IL-2 (106, 125). IL-2 was and is a very important mitogen for T cell cultures *in vitro* and has also been used in the clinic for immune therapy in cancer treatment and to treat HIV induced lymphopenia (126). It was thus a surprise that loss of IL-2 *in vivo* did not cause a major problem with T cell proliferation. IL-2 deficient mice develop a relatively normal lymphoid compartment, although, they did show elevated serum levels of IgG1 and IgE and suboptimal response when T cells were stimulated to proliferate *in vitro* (97). Never the less, initial studies showed the *in vivo* response to vaccinia and lymphocytic choriomeningitis virus (LCMV) was not impaired (127). The mitogenic role of IL-2 can thus be supplanted by other γc cytokines (128). However, the *in vivo* immune responses of IL-2 deficient mice are not completely normal and IL-2 has essential non redundant functions *in vivo* for the efficient production of effector cytotoxic T cells, and for sustaining T cell expansion and induction of tolerance (129-134). Most interestingly, IL-2-/- mice manifested autoimmune symptoms including autoimmune haemolytic anaemia and inflammatory bowel disease when allowed to age (135, 136). These diseases were associated with lymphadenopathy and accumulation of activated lymphocytes ultimately leading to autoimmunity. Similar phenotypes are also seen in the IL-2Rα-/- mice and in IL-2Rα deficient patients (137, 138). Thus it was obvious the role of IL-2 as a mitogen is redundant but that IL-2 plays a critical and non-redundant role in the maintenance of peripheral tolerance *in vivo*.

The mechanism by which IL-2 controls T cell homeostasis and maintains peripheral tolerance is not fully understood, but IL-2 is thought to elicit two

mechanisms in T cells that may play a pivotal role in tolerance; activation induced cell death (AICD), and homeostatic maintenance of CD4+CD25+ regulatory T cells (139-142). AICD is thought to be one of the processes contributing to cell death associated with the contraction phase of the immune response responsible for pathogen clearance. This massive cell death is thought to aid in eliminating activated T cells that may persist and mediate autoimmunity or malignancy. In vitro AICD is described as a sensitivity to cell death after exposure to primary activation signals, thus activated T cells will die rapidly if stimulated through the TCR multiple times.

AICD is mediated by the signalling events down stream of death receptors expressed on activated T cells. Death receptors associated with AICD are the tumour necrosis factor (TNF) receptor members, Fas and TNFR. Fas binds it's ligand, FasL, resulting in oligomerisation and recruitment of adapter molecule Fas-associated death domain protein (FADD), via binding of death domains (DDs) (143-145) FADD also contains a DD that interacts with the DDs on procaspase 8, which allows for the formation of a complex known as the death-inducing signalling complex (DISC) (146). Subsequent events downstream of a FasL induced DISC formation results in the activation of large amounts of caspase 8 (147), which then signals downstream to effector caspases 3 and 7, and leads to apoptosis. Caspase 3 has been shown to be important in AICD mediated apoptosis as T cells from caspase 3-deficient mice show a significant resistance to AICD and an improved survival after responding to superantigen, anti-CD3, anti-Fas antibody stimulation (148). Alternative apoptotic pathways initiated by DISC formation exist and are mediated by the pro-apoptotic Bcl-2

family member Bid (149). It is not clear how this pathway functions although it is thought that Bid interacts with pro-apoptotic proteins Bak, and Bax, to form pores in the outer mitochondrial membrane (150), releasing pro-apoptotic factors stored in the mitochondria, including cytochrome c (151-153).

Unstimulated naïve T cells are relatively resistant to AICD, as they express little to no FasL/Fas receptor. However, FasL has been shown to up regulate following TCR activation of naïve T cells, sensitising these cells to AICD. IL-2 has also been shown to up regulate FasL and promote apoptosis over proliferation in activated T cells (154). The importance of IL-2 for the induction of AICD is illustrated in IL-2 and IL-2Rα knockout mice that develop wide spread autoimmune disease, splenomegaly and lymphoproliferation, a result of unrestricted T cell proliferation (135, 155, 156). When IL-2 signalling was reinstated in IL-2/IL-2R knockout mice T cell susceptibility to AICD returned (157). It is thus proposed that IL-2 plays a role in sensitising T cells to AICD through the regulation of the Fas/FasL death pathway (156, 158).

One other critical role of IL-2 in immune homeostasis is its role in the growth and maintenance of CD4+CD25+ regulatory T cells (T regs) (131). Evidence has been mounting over the past few years in support of CD4+CD25+ regulatory T cell suppressive function (159, 160). It is now known that T regs are a subset of cells that develop in the thymus and migrate to the periphery where their main role is to suppress auto reactive T cells that have escaped negative selection in the thymus (141, 161, 162). T regs not only suppress autoimmunity but also control broad range of T cell dependent immune responses *in vivo*. For

example, transplant rejection, prevention of anti-tumour immunity and regulate the immune systems responses to infection (163). Although little is known about T reg generation, homeostasis, and inhibitory activity there is increasing evidence that IL-2/IL-2R signalling is central to these processes.
For example, transgenic mice deficient in IL-2, IL-2Rα, IL-2Rβ or the IL-2 activated transcription factor Stat5a/b are either missing or have decreased numbers of T reg's (164-169). It has also been noted that thymus restricted expression of IL-2Rβ restores functional T reg's in IL-2Rβ null mice suggesting that IL-2 signalling in the thymus is critical for the development of these cells (167). It has also been shown that when IL-2-/- mice are injected with a cellular source of IL-2 there is an increase in T reg numbers (165).

1.4.3 IL-9

IL-9 was initially identified as a late acting T cell growth factor and mast cell growth factor. IL-9 is a multifunctional cytokine secreted by Th2 lymphocytes, where it has been implicated in mast cell, eosinophil and T cell mediated pathologies, such as asthma (170-172). IL-9 deficient mice develop a normal lymphoid compartment but exhibit excessive mucus production and mast cell proliferation (173). IL-9 has growth factor and anti-apoptotic activities on multiple transformed cells suggesting a potential role in tumorigenesis. Indeed, it has been shown that IL-9 transgenic mice develop thymic lymphomas, consistent with the presence of IL-9 receptors in the thymus (174). Also, IL-9 is produced by Hodgkin disease and HTLV-I transformed T cells in humans (175-177). The IL-9R and common γc chains associate with JAK1 and JAK3, respectively, triggering STAT1, 3, 5, IRS and RAS-MAPK pathways. *In vitro*, a deregulated IL-

9 response can lead to autonomous cell growth and malignant transformation of lymphoid cells associated with constitutive activation of the Jak/STAT pathway.

1.4.4 IL-4

IL-4 has a key role in the immune system because it controls the development of T helper 2 cells. CD4+ T cells can be divided into T helper1 and T helper 2 (Th1/Th2) lymphocyte subsets based on their cytokine production profiles (178-182). CD4+ Th1 cells produce IFNγ, whereas CD4+ Th2 cells produce IL-4, IL-5, IL-9, IL-6 and IL-13. Cytokine production by CD4+ Th1/Th2 populations mediate the functional role for these cells *in vivo*, where CD4+ Th1 cells mediate immune responses against intracellular pathogens such as toxoplasma gondii and Leishmania and CD4+ Th2 cells are involved in antibody responses and protection against parasites such as intestinal helminths. IL-4 is crucial for Th2 polarisation of CD4+ T cells. For example, when IL-4, IL4R or Stat6 (the major Stat protein activated by IL-4) were deleted in transgenic mice, CD4+ Th2 differentiation was severely compromised (183-187).

1.4.5 IL-21

IL-21R is expressed in lymphoid tissues including spleen, thymus and resting peripheral blood cells like B, T, NK and dendritic cells (188-191). Both CD4+ and CD8+ T cells up regulate IL-21R on TCR activation (190, 192). Hence, the broad tissue distribution of IL-21R indicates a role for IL-21 signalling in both adaptive and innate immune systems. However, the expression of the cytokine IL-21 is not easily detected but is induced by phorbol myristate acetate (PMA) and ionomycin or anti-CD3/CD28 activated CD4+ T cells, and not in CD8+ (188).

Further analysis saw that only Th2 CD4+ T cells expressed IL-21 mRNA, *in vitro* and *in vivo* (193) and most of the data suggests this population of cells to be the primary source of IL-21, although others may yet be uncovered. Thus, because of this expression pattern, it has been suggested that IL-21 may play a role in the early differentiation of CD4+Th2 T cells.

Originally the IL-21 receptor (IL-21R) was identified by it's significant homology to the IL2Rβ subunit and the IL-21 protein cloned using a functional approaches (188, 189). IL-21R is expressed on T, B and NK cells and the cytokine (IL-21) is thought to regulate NK cell cytotoxicity/differentiation, B and T cell proliferation. Recent data shows that IL-21 acts synergistically with IL-15 in supporting proliferation of memory ($CD44^{Hi}$) and naïve ($CD44^{Low}$) CD8+ T cells and augments INFγ production *in vitro* (194). IL-21R-/- mice have normal T, B and NK cell numbers (195, 196), but were found to have diminished IgG1 production and elevated IgE.

The effect of IL-21 on T helper cell differentiation is still unclear. It has been reported that IL-21 is a Th2 cytokine (193) however it has also been seen to induce expression of Th1 related cytokines (197) and inhibit Th1 differentiation (193).

1.4.6 IL-15

Two independent groups originally identified IL-15 as a T cell mitogen, equal to IL-2, and able to induce T cell proliferation in the presence of IL-2 blocking antibodies (198, 199). The IL-15R consists of three chains: IL-15Rα, IL-2Rβ, and IL-2Rγc (200-202). IL-15 is the only member of the γc cytokine family that is known to signal through a receptor that shares two common subunits with that

of the IL-2 receptor. However, both IL-2 and IL-15 receptors are defined by the expression of a unique receptor-α subunit, IL-2Rα and IL-15Rα. The IL-2Rα subunit (CD25) is expressed on subsets of pre-B cells, thymocytes and subsets of activated mature B and activated T lymphocytes. IL-15Rα chain expression is far more ubiquitous, seen in lymphoid and myeloid cells, as well as non-haematopoietic cells (201). Both α-chains confer specific binding of IL-2 or IL-15 to trimeric receptors however there is no current biochemical evidence to show that the IL-2Rα and IL-15Rα subunits have signalling functions of their own. It is known that IL-15 is the only cytokine that binds to the IL-15Rα subunit with high affinity ($Ka=1x10^{11}\ M^{-1}$) in the absence of any other receptor chains. In addition IL-15 can also bind to IL-2Rβ and γc complexes with intermediate affinity ($Ka=1x10^{9}\ M^{-1}$) (203) allowing signals to be transduced via the IL-2Rβ/γc complex alone (104). IL-15 has also been found to bind to a unique receptor, IL-15RX, only expressed on mast cells (204). Because of the similarities between the IL-2 and IL-15 receptors and the fact they share common biological functions, it was always thought these two cytokines would also induce common biochemical and transcriptional programs. This view was supported by gene profiling experiments of γc cytokine stimulated peripheral blood T cells over short time courses. They found that IL-2, IL-15 and IL-7 regulated a very similar pattern of gene transcription (205). As well O'Shea and colleges showed that IL-2 and IL-15 induced a very similar pattern of tyrosine phosphorylation in T cells. For example both induced tyrosine phosphroylation of Jak1, Jak3, STAT5, and the IL-2Rβ chain (206).

One difference between IL-2 and IL-15 is that IL-2 is thought to function as a secreted soluble ligand whereas IL-15 signalling is proposed to occur as a *trans*

binding event whereby soluble IL-15 secreted by cells responding to inflammatory cytokines, binds to high affinity IL-15Rα subunits expressed on these cells and is presented in *trans* to IL-2β/γc complexes on recipient cells (207-212).

IL-15 is expressed in multiple tissues including placenta, skeletal muscle, kidney, heart, monocyte/macrophages and bone marrow stromal cells (199). The first cell types to be implicated as functionally relevant sources of IL-15 in the context of the immune system were members of the monocyte/macrophage lineage (213, 214). Blood derived dendritic cells have also been shown to produce IL-15 (215, 216).

IL-15 is a T cell mitogen *in vitro*, and can stimulate the proliferation of natural killer (NK) cells as well as supporting NK cell cytotoxicity. However, important *in vivo* differences in the action of these two cytokines have emerged particularly in regard to NK cell development and CD8+T cell homeostasis (202). As discussed, mice deficient for IL-2 and IL-2Rα show abnormal peripheral immune responses associated with lymphoproliferative disorders and the development of autoimmunity but have generally normal γ/δ T cell and NK cell development. However, mice lacking expression of the IL-2Rβ have an additional problem: profoundly decreased numbers of NK cells and γ/δ T cells. These data suggest that IL-15 and not IL-2 has a profound role in the development and differentiation of these cells (217).

Furthermore, mice lacking expression of either IL-15 or IL-15Rα do not have the autoimmune phenotype of IL-2 and IL-2Rα deficient mice but they lack the NK cell compartment, confirming a distinct role for IL-15 in NK cell development

(218, 219). IL15 or IL-15Rα mice also have decreased numbers of CD8+ T cells and almost a total lack of memory phenotype CD8+ cells, suggesting that IL-15 is critical for the homeostasis of naïve and memory CD8+ T cells (218, 219). Virus infection models concur with this showing that IL-15 is important for the generation and expansion of virus-specific effector CD8+ T cell clones (220, 221). The findings from IL-15 and IL-15Rα knockout animals are supported by the transgenic models, showing a role for IL-15 in the generation and proliferation of CD8+ T cell numbers (222), and over expression of a modified stable form of IL-15 mRNA causes CD8+ T cell lymphomas (223). IL-15 also plays a crucial role in the differentiation and maintenance of CD8+ T cells memory and is thought to be useful as a vaccine adjuvant for efficient generation of a memory T cell pool (218, 224) (208, 219, 220, 225-229).
Most T cell work with IL-15 has focused on its role in CD8+ T cell function but Geginat et al have suggested that IL-15 is a potent mitogen for different stages in CD4+ memory T cell differentiation (227, 230, 231). Congruently, although not as pronounced as the decrease in CD8+ T cell numbers, IL-15Rα/IL-15 -/- mice also showed a decrease in CD4+ T cell numbers. The importance of IL-15 for naïve and memory CD4+ T cell maintenance and development has not been fully worked out although it seems likely to contribute in some way (232, 233).

1.4.7 IL-7

IL-7 has effects on both T and B cell biology (234-236). The critical role IL-7 in T and B cell development was initially studied by injecting mice with antibodies to IL-7 and IL-7Rα (237), and then by studies of lymphoid development in mice deficient in IL-7Rα and IL-7 (238, 239), where T and B cell development was

abrogated in both cases. Patients with defective IL-7Ra expression have defective T and NK cell but normal B cell development (240), highlighting the non-redundant role of human IL-7Ra in T cell but not B cell development.

IL-7 is produced by thymic and bone marrow stromal cells (241-243). In the thymus, IL-7 dependent signals are critical at the double negative stage of T cell selection, as development is blocked at this stage in IL-7 and IL-7Rα deficient mice suggesting IL-7 dependent signals are required for normal cell division of DN and DP thymocytes.

IL-7 and IL7R null mice are also characterised by their reduced T cell numbers, in the thymus and periphery, and abnormal T cell response to polyclonal stimuli. Thus it is thought that IL-7 provides distinct signals that influence T cell survival and proliferation (234-236, 244, 245). In addition to mitogenic signals, the induction of the anti-apoptotic molecule Bcl-2 is induced by IL-7 (40). The earliest T lineage-committed cells thus depend on IL-7 for Bcl-2 expression survival and normal cell cycle progression (246). However, IL-7 signalling is not limited to Bcl-2 induction and is also thought to regulate TCR gene rearrangements (247) (248-250) (251).

The B cell defect in IL-7 and IL-7Rα deficient mice appears early in development (238, 239). Transgenic expression or injection of IL-7 augments the expansions of early B cells *in vivo* (252-254). The defects in B cell development seen in IL-7/IL-7R transgenic mice are thought to be due to a combination of effects on early B cell proliferation, survival and Ig gene rearrangement (236, 255).

A large body of evidence shows that IL-7 plays an important role in peripheral T cell homeostasis (40, 256-258). Homeostatic proliferation of naïve T cells is driven by the presence of 'space' (lymphopenia) in the host animal, such as that occurring in neonates (259, 260). This proliferation is also driven by the presence of low avidity self antigen: MHC (major histocompatability complex) interactions (261). Data strongly indicates that IL-7 plays a vital role in naïve T cell homeostatic proliferation. Hence naïve T cells in IL-7 deficient hosts have a reduced proliferative rate but their proliferation is increased when IL-7 is over expressed in mice (262-264). IL-7 has also been implicated in the expansion, survival and homeostatic proliferation of CD8+ memory T cells (39, 258, 262, 265). Indeed, IL-7 receptor expression is thought to flag effector CD8+ T cells with the potential of becoming memory cells (266, 267). IL-7 has also been implicated in the formation and maintenance of CD4+ memory T cells (263, 268). However, it is not thought that IL-7 is the sole survival factor for memory CD8+ T cells as a large body of evidence implicates a vital role for IL-15 (200, 269, 270).

1.5 Cytokine receptor signal transduction

The γc cytokine receptors have no intrinsic catalytic activity yet studies showed that cytokine binding induced immediate tyrosine phosphorylation of cellular proteins and receptor subunits (**Fig 1.5.1**). The key tyrosine kinases for the γc cytokine receptor family are the Janus kinases Jak1 and Jak3. The isolation and cloning of the Jak tyrosine kinases was first established in the context of Interferon signal transduction (reviewed in (271-273)). There are four different Jaks: Jak1, Jak2, Jak3 and Tyk2. Jak1, Jak2 and Tyk2 are ubiquitously and

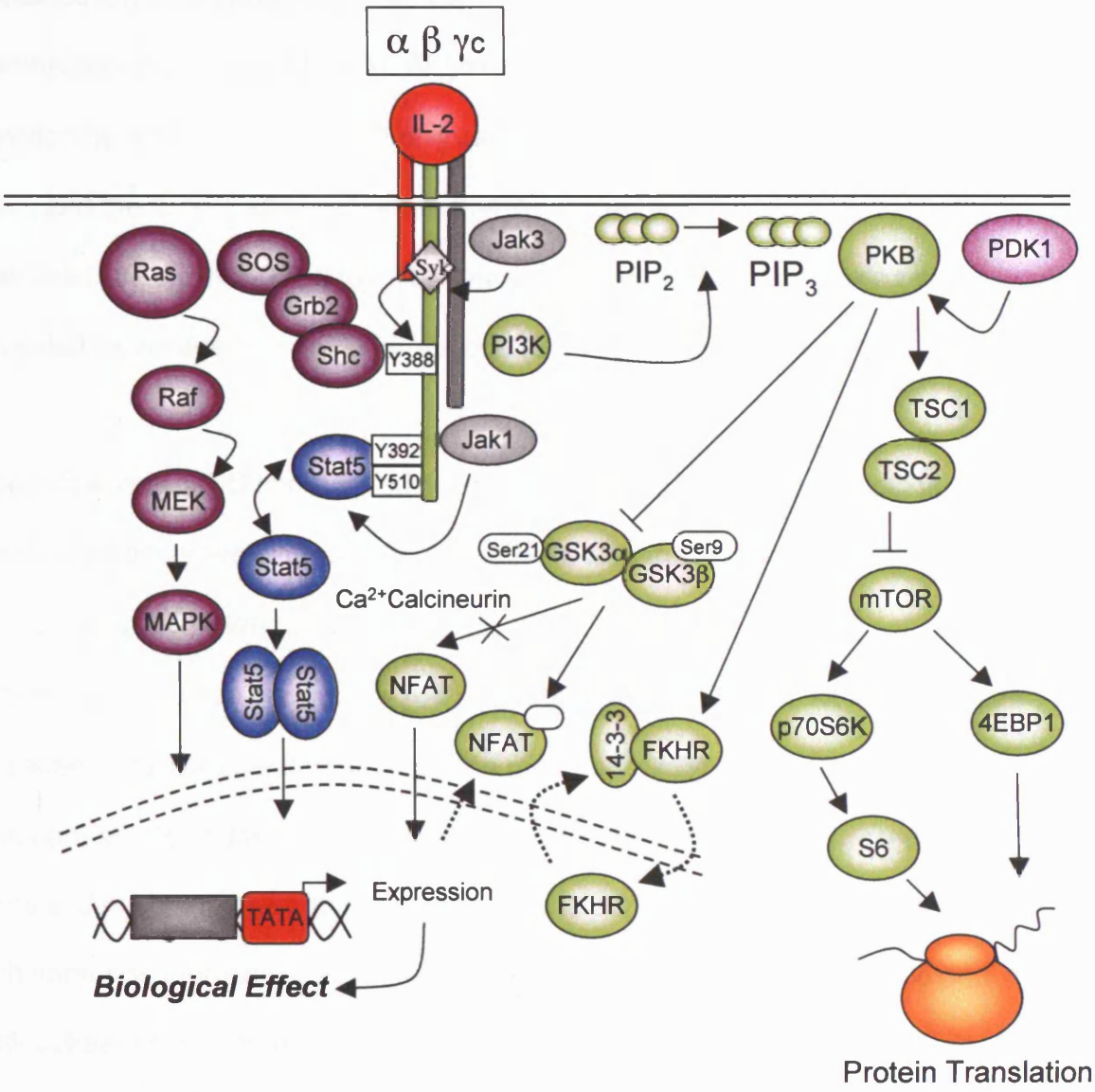


Figure 1.5.1: Signal transduction down stream of IL-2 receptor binding. Cognate cytokine binding brings together receptor subunits, resulting in activation of tyrosine kinase activity, association of Jak's, dimerisation of STAT proteins and transcription of target genes in the nucleus. PI3K associates with IL-2Rβ via phospho-tyrosine residues, triggering PIP_3 production and activation of down stream pathways involving Akt (PKB) which feeds into metabolically active pathways such as glycolysis (GSK3), protein synthesis and cell growth (mTOR). The MAP Kinase pathway has also shown to be activated down stream of IL-2 binding via a Ras mediated pathway. *Y: phosphorylated tyrosine residue*

constitutively expressed, although Jak3 expression is restricted to haematopoietic cells (273, 274). All γc containing cytokine receptor complexes function by activating both Jak1 and Jak3. The common γ-chain associates with Jak3 and the IL-2Rβ chain whereas IL-4Rα, IL-9R, IL-7Rα and IL-21R associate with Jak1 (102, 204, 275, 276). The analysis of the human Jak3 gene has revealed its linkage to a SCID immunodeficiency (85) (275, 277, 278).

The autosomal SCID immunodeficiency's associated with Jak3 mutations cause identical phenotypes to those seen in patients with the γc subunit mutation or X-linked immunodeficiency SCID (87, 102, 279).
Indeed, Jak3-/- mice phenocopy IL-2Rγ-/- mice (280-282). This similarity occurs because γc recruits Jak3 to the cytokine receptor complex.
The central role of Jaks in cytokine signalling makes them important therapeutic targets. Jak3 is the most recent focus for generating a new class of inhibitors with immunosuppressive, anti-inflammatory, anti-allergic, anti-thrombotic and anti-leukaemic properties and is in preclinical studies (283).

1.5.1 Signalling downstream of Jak kinases

Substrates down stream of Jak1 and Jak3 have not been fully characterized, but are known to include tyrosine residues in the specific chains of cytokine receptors. For example, IL-2 activation of the Jaks results in phosphorylation of three tyrosine residues on the IL-2Rβ chain, which are vital for signal transduction: Tyr338, Tyr392 and Tyr510 (273). Activation of Jaks also results in tyrosine phosphorylation of adapter molecules such as Gab2, Shc, IRS-1, Grb2, SHP-2, Vav and STAM (206, 284-289). These tyrosine phosphorylation events

induced by γc cytokines activate at least three known signalling pathways involving Ras/MAP Kinases, signal transducers and activators of transcription (STATs) and PI3K (290).

1.5.2 Ras proteins are activated by a subset of γc dependent cytokines.

Immunological interest in GTPases stems from observations in the early 1990's that the GTPase Ras accumulates in the active GTP bound state in response to antigen receptor ligation or in response to γc cytokines such as IL-2. Two main classes of regulatory proteins control Ras activity: guanine nucleotide exchange proteins (GEFs) that promote the transition from the inactive GDP-bound to the active GTP-bound conformation and GTPase activating proteins (GAPs) that stimulate GTPase inactivation. In IL-2 signalling it has been shown that phospho-Tyr388 recruits an adapter molecule Shc. The best-defined role for Shc is the activation of the guanine nucleotide binding protein Ras (291). In response to IL-2, Shc binds to phospho-Tyr338 of the IL-2Rβ chain and becomes tyrosine phosphorylated (292, 293). Tyrosine phosphorylated Shc then is able to bind to the SH2 domain of a second adapter Grb2. The Ras guanine nucleotide exchange protein Sos, mammalian homologue of the Drosophila 'Son of Sevenless' protein, associates constitutively with the src homology (SH3) domains of Grb2. The Shc/Grb2 complex formed in IL-2 activated cells results in the membrane re-localisation of Sos enabling the catalytic activity of Sos to elicit Ras activation.

Interactions between Ras GAP and tyrosine phosphorylated SOCS3 proteins have also been described in the context of IL-2 signal transduction. The

formation of these protein complexes might either allosterically inhibit GAP functions or sequester this negative regulator and physically prevent interaction with Ras proteins thereby promoting activation of Ras (294).

Once Ras is activated it is able to regulate diverse cellular processes by coupling to multiple biochemical effector signalling pathways including the Raf-1/MEK/Erk1, 2 kinases (295, 296). The activation of this pathway is needed for γc cytokine induction of cell cycle progression (297), however, the role of Ras as a mediator of cytokine signalling in the context of T cell differentiation has not been explored. Interestingly, not all γc cytokines activate Ras. The activation of the SHP and Ras/MAP kinase pathway is common to IL-2 and IL-15 (206, 298). In contrast, IL-7 does not induce Shc tyrosine phosphorylation or Erk activation (297, 299), and IL-4 does not induce Ras activation (298, 300, 301).

1.5.3 STAT proteins are activated by γc dependent cytokines

Key transcription factors that mediate cytokine responses are known as signal transducers and activators of transcription (STATs). Seven mammalian STAT proteins are known, Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6. Biochemical and molecular analysis revealed Stats are direct substrates of Jak kinases and are tyrosine phosphorylated in cytokine-activated cells (274, 302). All STAT proteins have a conserved COOH SH2 domain and a COOH tyrosine phosphorylation site. Following Jak mediated tyrosine phosphorylation; STATs became dimerised through phosphotyrosine-SH2 domain interactions. STATs can form homodimers as well as heterodimers. Latent STAT proteins are predominantly cytoplasmically localised. An immediate consequence of receptor triggering is JAK activation and tyrosine phosphorylation of the cytoplasmic

domain of the receptor subunits. In the prototypic model, STAT activation is initiated by the recruitment of STATs to tyrosine-phosphorylated sites within the activated receptor subunits. JAK mediated tyrosine phosphorylation then occurs which allows for SH2 mediated STAT dimerisation. Active dimerised STATs translocate into the nucleus where they bind to high affinity DNA motifs containing the consensus palindromic inverted repeat sequence TTC(N_{3-4})GAA. Each individual STAT has particular DNA binding affinity for specific sequences. This depends not only on the STAT DNA binding domain itself but also on the different homo and heterodimer combinations that it forms. STAT dimers can also polymerise through mutual N-terminal interactions to bind DNA co-operatively, enabling the recognition of variations of the consensus site .The conserved COOH terminus domain acts as a transactivation domain (TAD).

The immediate biochemical consequences of triggering T cells with many different γc cytokine results in a common response: activation of the cytosolic tyrosine kinases, the Janus kinases Jak1 and Jak3. However, differences in the cytosolic domains of cytokine receptor subunits, which act as intracellular adapters for recruitment of signalling molecules, can thereafter allow immediate divergence of signal transduction pathways. For example γc cytokines IL-2, IL-7 and IL-15 activate a common transcription factor STAT5 whereas IL-4 activates STAT6. This difference occurs because the IL-2Rβ subunit and the IL-7Rα subunit contain tyrosine residues Tyr392, Tyr510 and Tyr449 respectively (303, 304) that act as docking sites for STAT5 whereas tyrosines in the IL-4 receptor subunit act as docking sites for STAT6 (305).

1.5.3 The importance of the STATs for cytokine signal transduction.

Mice deficient in expression of the various STATs have been made and studies of these mice have revealed that the STATs are important regulators of haematopoietic cells and play a role in regulating the immune system. For example, STAT6 appears to be crucial for the function of the γc cytokines IL-4 and IL-13. Stat6-deficient mice thus have a similar phenotype to IL-4 or IL-4Rα -/- mice, displaying defective Th2 lineage T cell differentiation and defective B cell Ig class-switching (183-186). Stat3 and Stat1 are also activated by γc dependent cytokines: STAT3 is activated by IL-2, -7, -15, -9 and -21 (105, 304, 306, 307) (308). STAT1 can be activated by cytokines IL-2, IL-7, IL-15, IL-12 and IL-6 (309, 310); however, STAT1 was initially discovered as a result of interferon signal transduction (311, 312).

STAT3 deficiency is embryonic lethal, resulting from defects in cytokine signalling outside the haematopoietic system (313). T cell specific knockouts of STAT3 develop normally and have normal IL-7 mediated T cell proliferation, but are partially defective in IL-2 induced proliferation arguing that STAT3 may be involved in IL-2 signal transduction (314). STAT1 deficient mice have an impaired response to IFN α/β/γ, poor anti-viral responses, and develop spontaneous and chemically induced tumours more rapidly than wild type mice. Suggesting that STAT1 may not be a crucial transactivator for γc dependent cytokine signalling (315) (316).

The predominant STATs activated by γc cytokines are STAT5a and STAT5b but these STATs are also activated by other cytokines important for haematopoiesis

and indeed Stat5 was originally identified as a transcription factor that regulates the β-casein gene in response to prolactin (317).

Mice deficient in Stat5a and/or Stat5b thus have a complex phenotype reflecting the involvement of these transcription factors in signalling pathways regulated by a diverse set of cytokines and growth factors in different cell types (318). Hence it is difficult to correlate the significance of STAT5 activation to one particular T cell function or defect in γc cytokine signal. However, even though mice lacking Stat5a have a small but significant decrease in splenocyte numbers and normal lymphoid development, it has been reported that T cells in these mice express less IL-2Rα (CD25) chain after *in vivo* antigenic stimuli with staphylococcal enterotoxin B (SEB) (319). These cells were also shown to have impaired IL-2 induced proliferation (at low concentrations of IL-2) and a sub-maximal response *in vivo* antigenic stimuli, in part reflecting that STAT5a seems to be required for maximal expression of CD25 and thus T cell proliferation (319). The immunological phenotype of Stat5b-/- mice is more severe than that of Stat5a-/- mice, where thymocyte numbers are slightly reduced, peripheral T cell numbers are more reduced than Stat5a-/- mice, NK cell numbers and IL-2 induced cytotoxicity are reduced (320). These data argue that Stat5b maybe functionally more important than Stat5a for T and NK cell function (319, 320). As expected, Stat5a/b double knockout mice show a more severe phenotype than either of the single knockout mice. Where T cells respond poorly to antigen stimulation, even in the presence of high IL-2, develop splenomegaly and have T cells with an activated phenotype, in part suggesting defective IL-2 signalling. These mice also lack NK cells, which is consistent with defective IL-15 signalling

although taken together the phenotype recapitulates some of the symptoms seen in IL-2Rβ deficient mice.

There is also evidence that STAT5 is necessary for IL-7 mediated responses during both B and T cell development. For example, peripheral blood B cells of Stat5a/b deficient mice are significantly reduced, and pre and pro-B cells were found to have a reduced response to IL-7 (321, 322). As well, Pallard et al showed that a dominant negative STAT5 mutant blocked thymocyte differentiation in foetal thymic organ cultures (FTOC) (323).

In vitro and *in vivo* data from cell lines and mice models also indicate that Stat5 is important for IL-2 dependent T cell proliferation (293, 322, 324). Moreover, constitutive expression of Stat5 in cell lines results in T cell proliferation and constitutively active STAT5 has been isolated from HTLV-1 transformed T cells, B and T cell lymphomas (304, 325-328). Indeed, data suggests that expressing constitutively active STAT5a in naïve CD4+ T cells can independently drive Th2 differentiation of CD4+ T cells in the absence of IL-2, STAT6 and IL-4Rα, suggesting that STAT5a plays an important role in CD4+ differentiation (329).

The direct gene targets for STATs down stream of γc dependent cytokine receptor signals, has not been well studied. There has been recent data suggesting STAT5 proteins control pro-mitogenic and oncogenic genes including IL-2Rα, Pim1 and the D type cyclins (D1 and D2) (330-336). However, much more work is needed in this area to understand the role of identified and unknown targets in γc dependent cytokine signalling.

1.5.4 γc dependent cytokine activation of PI3K

A major route for inositol lipid metabolism initiated by γc dependent cytokines is mediated by phosphoinositide-3 kinases (PI3K) The role of PI3K is to phosphorylate $PI(4,5)P_2$ at the D3 position of the inositol ring of inositol phospholipids producing $PI(3,4,5)P_3$ (337).

There are multiple isoforms of PI3Ks in mammalian cells (see (338-340) for reviews). The forms linked to γc dependent cytokine are the Class I PI3Ks, comprising a heterodimer of a catalytic subunit p110 and a regulatory/adapter subunit p85.

The initial model for PI3K activation by γc cytokines was patterned on the prototypical model worked out for growth factors in the context of receptor tyrosine kinases in fibroblasts. In this model receptor tyrosine kinases phosphorylate stimulated receptor subunits. The PI3K p110 catalytic subunit is recruited to the receptor via high affinity binding between the Src homology (SH) 2 domain of the p85 adapter subunit and specific phosphorylated sequences within the cytoplasmic tail of the receptor (341, 342). This process recruits the p110 catalytic subunit of PI3K to the plasma membrane where it can phosphorylate its main substrate $PI(4,5)P_2$ to generate $PI(3,4,5)P_3$. The ability of p85 to bring the catalytic subunit to the plasma membrane is a crucial role for this adapter; constitutive membrane targeting of p110 catalytic subunits of PI3K creates an enzyme that constitutively generates $PI(3,4,5)P_3$ and $PI(3,4)P_2$ when expressed in cells (82). PI3K signalling in response to γc cytokines initially seemed to fit a simple model whereby Jaks mediated tyrosine phosphorylation of receptor subunits (Tyr392 in the IL-2Rβ chain, Ty449 in the IL-7 receptor subunit or Tyr731 in the IL-4 receptor) initiating p85 mediated recruitment to the

plasma membrane (343-345). However, PI3K recruitment to the plasma membrane is not necessarily mediated by direct receptor binding, there are a number of reports of interactions between p85 and other adapters that could bring p110 to the plasma membrane in T cells. For example, a complicated signalling scaffold formed by three adapters, Shc, Grb2 and Gab2 recruits PI3K to the plasma membrane in cells activated by haematopoietic cytokines such as IL-2 and IL-3 (206, 346).

As discussed, multiple isoforms of PI3K exist and are co-expressed in T cells. It is not yet known which PI3K isoforms are regulated by γc cytokines in T cells although the *in vivo* biological role of different PI3K isoforms has begun to be elucidated through the generation of knockout mice models by gene targeting methods. A full discussion of the phenotype of mice lacking the different PI3K subunits is beyond the scope of the present report and has been extensively reviewed (80, 347). It should be emphasised that work involving lymphocyte function in PI3K knock out models has focused on TCR/CD28 mediated signals, development, proliferation and migration and virtually no work has looked directly at the impact of losing PI3K isoforms on signalling down stream of IL-2 receptors and other γc cytokines IL-7, IL-4, IL-15, IL-9 and IL-21.

It is however worth discussing the phenotype of mice deficient in expression of p110δ, which is selectively, expressed by haematopoietic cells. Here Okkenhaug and colleagues generated mice expressing a catalytically inactive form of the p110δ subunit (p110δ $^{D910A/D910A}$) referred to as p110δ knock-in mice (81). These mice were fertile and healthy. T cells in the thymus appeared normal although T cell numbers in the spleen were reduced. Peripheral T cells

expressed higher than normal levels of naïve markers CD44 and CD62L, seeming more 'naïve' than controls, and had a reduced signalling capacity and IL-2 production when responding to antigen presented by B cells. Hence, it is suggested that T cells develop normally in these mice although peripheral maturation and survival is disrupted. Over time p110δ $^{D910A/D910A}$ mice developed inflammatory bowel disease, a complex autoimmune response, thought to be mediated by dysfunctional regulatory T cells. The deregulation of immune tolerance in these mice may suggest impaired γc cytokine signalling as uncontrolled proliferation and autoimmunity has been seen in IL-2/IL-2R-/- mice. The B cell defect in these p110δ $^{D910A/D910A}$ mice very severe, where B cell numbers are largely reduced, proliferation inhibited and apoptosis accelerated. This B cell phenotype is similar to P110δ-/- mice created by Clayton *et al* (348) using a gene deletion protocol. These results have been interpreted as evidence that P110δ maybe important for PI3K signalling down stream of the B cell receptor (BCR) (348, 349) (350, 351).

There is evidence that PI(3,4,5)P_3 functions in γc signal transduction. Firstly, experiments *in vitro* with PI3K inhibitors such as LY294002 or wortmanin have shown a role for PI3K in γc cytokine mediated cell cycle progression, T cell differentiation and anti-apoptotic function (352-360) (323, 361-367). Conversely, elevation of cellular levels of PI(3,4,5)P_3 in T cells lines *in vitro* by expression of constitutively active membrane targeted PI3K can substitute for γc cytokines in the regulation of the E2F family of transcription factors (357). Studies of phospholipid phosphatases *in vivo* have also highlighted the importance of PI(3,4,5)P_3 signalling in γc cytokine signalling. Hence, PI(3,4,5)P_3 is normally

destroyed by a 3 ' inositol lipid phosphatase PTEN (phophatase and tensin homologue deleted on chromosome 10) (368) which mediates the conversion of PI(3, 4, 5)P_3 to PI(4, 5)P_2. Deletion of PTEN results in constitutive accumulation of PI(3, 4, 5)P_3 with severe consequences. Complete gene inactivation of PTEN in mice is embryonic lethal (369). PTEN is also tumour suppressor gene in humans and is located in a region of the chromosome that suffers loss of heterozygosity in many human cancers. In this respect, mice that have lost a single PTEN allele have complicated phenotypes associated with increased tumorigenesis and the development of polyclonal autoimmune disease (370). Recently, T cell specific knockouts of PTEN in mice have shown that PI(3,4,5)P_3 concentrations play a pivotal role in thymocyte proliferation and development. In particular, the thymocyte developmental defects seen in IL-2Rγc-/- mice can be rescued when by simultaneous deletion of a single allele of PTEN in pre-T cells i.e. the role of γc cytokines in the thymus can be fully substituted by elevating PI(3,4,5)P_3 cellular concentrations (371). There is one caveat about interpreting the PTEN rescue experiments namely that there is evidence that in certain circumstances PTEN has protein kinase activity (372).

1.6 Downstream of PI3K in T cells

PI(3,4,5)P_3 and PI(3,4)P_2 bind to pleckstrin homology (PH) domains of proteins (373). This allows for allosteric modification of activity or re-localization of the protein to defined areas of the plasma membrane for further signal transduction. Three major classes of signalling molecule are regulated by D3 phosphoinositide binding to PH domains: guanine nucleotide exchange proteins for Rho family GTPases, the TEC family tyrosine kinases such as Btk and Itk in

B and T lymphocytes respectively and the AGC super family of serine/threonine protein kinases (**Fig 1.6.1**).

1.6.1 PI3K regulation of guanine nucleotide binding proteins

Guanine nucleotide binding proteins cycle between a guanosine diphosphate (GDP)-bound or guanosine triphosphate (GTP)-bound state. These proteins function as binary switches controlling activation in response to environmental cues. GTPases exist in two different conformations when bound to GTP or GDP. The GTP-bound state is the active form allowing GTPases to interact with a down stream signalling cascades and mediating biological outcomes. Members of the Ras family of GTPases, Ras, Rap1A and the Rho family of GTPases Cdc42, Rac1, Rac2 and RhoA are important molecules for T cell signal transduction (74). Activation of Rac and Rho is stimulated by guanine nucleotide exchange proteins (GEFs) which characteristically comprise a catalytic Dbl homology domain flanked by a PH domain that is critical for GEF function, for review see (374). In particular, GEFs involved in activation of the GTPase Rac-1 have a PH domain that can bind $PI(3,4,5)P_3$ with high affinity (375). Rac is now known to mediate signals from PI3K down stream of IL-2 receptor signals (361): IL-2 regulates T cell shape and motility, along with T cell proliferation, and is thought to provoke membrane ruffling via a PI3K/Rac pathway (361).

1.6.2 PI3K and tyrosine kinases

The TEC family of tyrosine kinases, Itk, Tec and Btk are activated via a phosphorylation event within their activation loop by src kinases (376, 377). Most TEC kinases contain a plextrin homology (PH) domain that binds

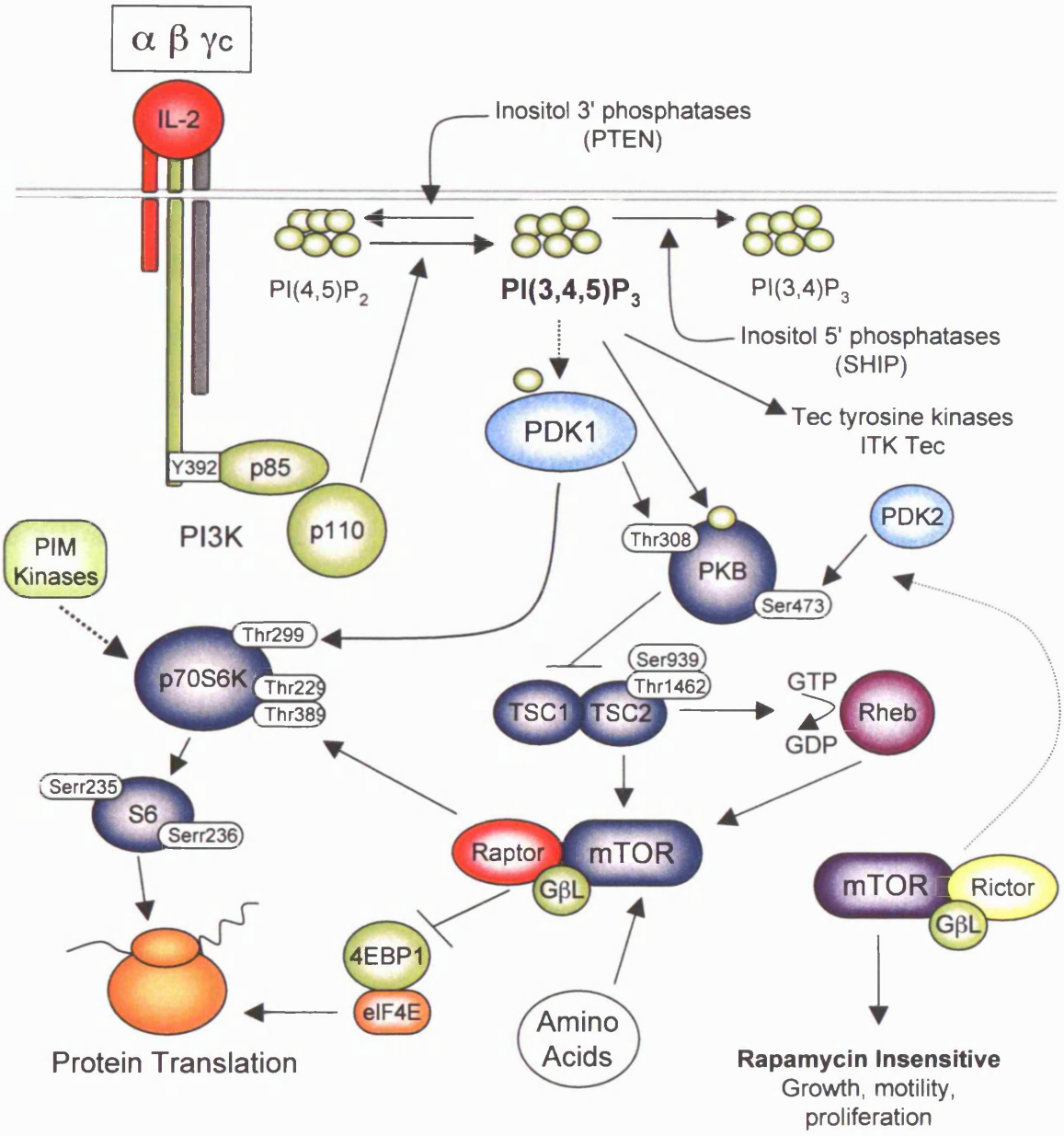


Figure1.6.1: PI3K signaling and protein translation initiated by IL-2/IL-2 receptor binding. Receptor dimerisation and association of PI3K p85 subunit catalyses $PI(3,4,5)P_3$ production. PIP_3 brings PKB to the membrane leading to activation by PDK1. Active PKB is known to regulate glucose metabolism and protein synthesis machinery through GSK3 and mTOR/p70S6K respectively. mTOR promotes p70S6K activation of ribosomal protein S6 and de-represses 4EBP-1 binding to translation initiation factor eIF4E promoting mRNA association with polysomes and thus protein synthesis. It is now thought that protein synthesis is regulated through rapamycin sensitive (mTOR/Raptor) or insensitive (mTOR/Rictor) pathways.

$PI(3,4,5)P_3$ and translocates the TEC kinases to the plasma membrane for close proximity to Src kinases for activation (378, 379). The importance of the PH domains of these kinases for their activation is illustrated in the context of BTK, where mutations in the BTK PH domain cause of X-linked agammaglobulinaemia in humans and a similar x-linked immunodeficiency in mice (380-383). BTK kinases are activated in response to cytokine receptors. Not much is known about Tec kinases in γc cytokine signalling although it has been shown that IL-2 regulates the activity of the tyrosine kinases Itk and Tec via activation of PI3K (384).

1.6.3 PI3K and serine/threonine kinases

A serine/threonine kinase that mediates PI3K action is protein kinase B (PKB) or Akt (385, 386). PKB has a PH domain that can bind $PI(3,4,)P_2$ and $PI(3,4,5)P_3$ *in vitro*. However it is thought that PKB preferentially binds $PI(3,4,5)P_3$ *in vivo*, because reduction of $PI(3,4,5)P_3$ levels by inositol phosphatase SHIP, and a concomitant increase in $PI(3,4,)P_2$, does not favour PKB activation (387, 388). The elevation of $PI(3,4,5)P_3$ levels in T cells seems sufficient to activate PKB. For example membrane targeting of the PI3K p110 catalytic subunit, which results in constitutive production of $PI(3,4,5)P_3$, is sufficient to trigger PKB activation (388, 389). Similarly, elevation of $PI(3,4,5)P_3$ levels in PTEN null cells results in constitutive activation of PKB and an uncoupling of PKB activation from mitogenic stimuli (368, 390).

PKB activation is regulated by the upstream kinase PDK1 (phosphoinositide-dependent protein kinase 1) that acts down stream of PI3K/$PI(3,4,5)P_3$ and

phosphorylates PKB on Thr308 in the kinase activation loop. Thr308 phosphorylation is thought to relieve auto inhibition of the PKB catalytic domain and is considered to be a key step in PKB activation (391-397).
PKB is also phosphorylated on Ser473 in a hydrophobic motif at the enzymes carboxyl terminus by a kinase known as PDK2 (398, 399). Recent data suggests that PDK2 is the target of rapamycin kinase (TOR) and its associated protein Rictor (400).

The importance of PDK1 for PI3K action was first established in the context of PKB: but it is now recognised that PDK1 is constitutively active and phosphorylates key residues in the activation loop or 'T loop' of probably all serine/threonine kinases of the AGC superfamily including all Protein Kinase C isoforms (401, 402), the ribosomal S6 Kinase, S6K1 (p70S6 kinase) (396, 403) and p90Rsk (395). PDK1 has an N-terminal catalytic domain and a C-terminal PH domain that binds $PI(3,4,5)P_3$ with high affinity. The role of D3 phosphoinositides in controlling PDK1 activity and subcellular localisation is very controversial (404, 405) (For review see (403, 406)). One view is that PDK1 specificity is regulated primarily by substrate conformation (403, 407) whereby, the D3 phosphoinosotides act to change the structure of PDK1 substrates at the membrane facilitating PDK1 mediated activation, rather than directly stimulating PDK1 (408). It has also been suggested that D3 phospholipids initiate PH domain dependent re-localisation of PDK1 from the cytosol to the plasma membrane (404, 408). Initial work in mouse embryonic stem cells lacking both copies of the PDK1 gene has allowed for the analysis of down stream pathways of PDK1. From this data it was clear that PDK1 interacted with multiple

members of the AGC kinase family in different ways, specifically PKB, were it was clear that multiple phosphorylation events were required for full activation *in vivo* (395).

Transgenic mice that lack PDK1 or have hypomorphic alleles, which express only 10% of normal levels of PDK1, have been produced. PDK1-/- embryos die at day 9.5, however hypomorphic PDK1 mice are viable and fertile but with 40-50% smaller body size than controls. One important observation was that cell numbers in these mice remained the same but organ size was smaller, hence the volume of cells lacking PDK1 was reduced 35-60% of normal suggesting PDK1 plays a pivotal role in regulating cell size independently of proliferation (409).

Recent data from Hinton et al shows that PDK1 is an essential kinase for T cell development as the loss of PDK1 in T cells blocks T cell differentiation in the thymus (410). Hence analysis of the PDK1 hypomorphic mice and mice with a T cell specific knockout of PDK1 genes indicated that complete loss of PDK1 in T cells blocked T cell differentiation in the thymus and reduced PDK1 expression allowed T cell differentiation but blocked proliferation (410).

1.7 PKB function in lymphocytes

1.7.1 Glycogen Synthase Kinase 3 (GSK3)

The first known substrate for PKB was Ser21 in GSK-3α and Ser9 in GSK3β (411, 412). These phosphorylation events have been shown to occur in lymphocytes responding to TCR activation and IL-2 stimulation (78, 413) and are associated with inactivating GSK3.

GSK-3 was initially identified as a regulator of glycogen metabolism but may have a much broader range of functions. Including phosphorylation of transcription factor Nuclear Factor of Activated T cells (NFAT) in T cells. NFAT is controlled by a phosphorylation/de-phosphorylation cycle: phosphorylated NFAT are mainly cytoplasmic and de-phosphorylated forms accumulate in the nucleus (414-416). NFAT de-phosphorylation is mediated by calcium phosphatase calcineurin, promoting NFAT nuclear import and transcription of early immune response genes, including cytokines (414, 417). In contrast the phosphorylation of NFAT by kinases including GSK-3 drives nuclear export (413). Thus PKB phosphorylation of GSK-3 inactivates the kinase limiting re-phosphorylation of NFAT and promoting de-phosphorylated NFAT nuclear retention. Gene targets of NFAT include TNFα, IL-2 and IL-4 suggesting a role for PKB in regulating immune responses (415). Although the role of PKB in NFAT regulation has not been directly been looked at there is evidence that expression of an active mutant of PKB in T cells of transgenic mice promotes T cell mediated inflammatory immune responses (418). Additionally PKB can substitute for CD28 co-stimulation of IL-2 and IFNγ gene transcription although the later response may rely on PKB-NFκB/Rel family transcription factors and not NFAT (419, 420). PKB does not mediate all CD28 functions but NFκB control of IL-2 and IFNγ genes by antigen receptors and CD28 is controlled synergistically by PKB and PKCs (420, 421). The actual substrates for PKB in NFκB regulation in lymphocytes are not known.

1.7.2 Forkhead transcription factors

PKB phosphorylation and regulation of the FOXO transcription factors is an evolutionarily conserved pathway. The FOXO family of transcription factors includes: FOXO1a (FKHR), FOXO3a (FKHR.L1) and FOXO4 (AFX) (422, 423). PKB phosphorylation of FKHR, FKHRL and AFX promotes their export from the nucleus to the cytoplasm where they can form a complex with 14-3-3 proteins, which retains them in the cytoplasm away from target gene promoters. Thus the model for PKB/FOXO regulation of gene expression is that in quiescent cells FOXO proteins are in the nucleus where they induce transcription of genes encoding pro-apoptotic proteins such as the Bcl-2 family member Bim and Fas ligand. FOXO proteins also induce transcription of cell cycle inhibitors such as p27Kip1 (424) (422, 425) PKB mediated nuclear export of FOXO transcription factors prevents their transcriptional activity and is associated with increased cell survival and cell cycle progression (424).

Recent insights about the importance of FOXO transcription factors for lymphocytes has come from studies of mice lacking FOXO3a (FKHRL1), the predominant forkhead protein expressed in peripheral lymphoid organs. These mice develop spontaneous lymphoproliferation and a complex organ inflammation disease that correlates with deregulated apoptosis in T cells. The result is hyper activated helper T cells, which proliferate rapidly and produce excess Th1 and Th2 cytokines (426).

1.7.3 PKB and cell survival

One of the most highly publicised functions of PKB is in cell survival (427-430). Most of the work ascribing the anti-apoptotic function to PKB has been in

fibroblasts and epithelial cells. In these cells PKB mediated prevention of cell death has been linked to the phosphorylation and inactivation of proapopotic protein Bad. PKB has also been linked to suppression of Fas-mediated cell death (431). Most of the work defining PKB as a survival factor has been worked out in epithelial cells although Craig Thompson's lab has shown that IL-3 induction of PKB activity is linked to survival in pre-B cells (432). In the pre B cell model PKB regulation of glucose metabolism via regulation of glucose transporters is a key survival signal (433).

There is thus a model whereby the ability of PI3K/PKB to control lymphocyte survival is mediated by PKB control of glucose metabolism (434-436).

A role for PKB in regulating glucose metabolism in T cells has also been proposed in the context of CD28 signal transduction (437). There is some evidence that T cells from transgenic mice expressing constitutively active PKB under control of a T cell specific promoter show enhanced survival and have enhanced rates of glycolysis (436, 438). However, PI3K inhibitors preventing PKB activity cause T cells to arrest in G0/early G1 of the cell cycle without apparent loss of cell viability (357). Similarly other studies do not see a correlation between PKB activation and cell survival in haematopoietic cells (439) (440). Thus, it would seem that Akt/PKB is not an essential regulator of cell survival in T cells. Moreover it is likely that $PI(3,4,5)P_3$ binding to proteins other than PKB is important in T cells. For example active PKB mutants cannot substitute for γc cytokine signalling during thymocyte development whereas loss of the inositol phosphatase PTEN which causes accumulation of $PI(3,4,5)P_3$ does rescue thymic developmental defects in γc null mice (371).

The mechanism by which γc cytokines regulate cell survival not fully been worked out. It has been shown that IL-2 inhibits expression of the pro-apoptotic molecule BAD but induces expression of anti-apoptotic molecules Bcl-2 and Bcl-XL (428, 441-444) but PKB may not be the sole regulator of these responses.

1.7.4 PKB substrate TSC: a link to protein synthesis and nutrient sensing

Tuberous sclerosis factors TSC1 (harmartin) and TSC2 (tuberin) form an inhibitory heterodimer complex known to be instrumental in regulating cell size and proliferation (445-448). These proteins form an evolutionarily conserved link between PKB and the nutrient-sensitive (mammalian target of rapamycin) mTOR pathway (reviewed in (449)). Akt/PKB phosphorylates TSC2 on Ser939 and Thr1462; additional phosphorylation sites include Ser981, Thr993, Ser1130, Ser1132, and Thr1162 (450-453). Growth factor induced TSC2 phosphorylation is sensitive to PI3K inhibitors wortmannin and LY294002 (450, 451, 453) and can be induced by expression of constitutively active PI3K or Akt/PKB (451, 454). TSC2 phosphorylation by PKB is functionally important for the activation of the ribosomal S6 kinase 1 (S6K1) by mTOR kinases (see below for further discussion (450)).

1.8 mTOR

Signal transduction by PKB is linked to the TOR kinases (also known as FRAP, RAFT, or RAPT). These are large, evolutionarily conserved serine/threonine kinases, related to PI3Ks, that play a central role in integrating signals from nutrients (amino acids and energy) and growth factors (in higher eukaryotes) to

regulate cell growth and cell cycle progression (reviewed in (455)). mTOR stands for mammalian target of rapamycin, as the TOR kinases were discovered from studies of the mechanism of action of the immunosuppressant rapamycin. Rapamycin is a natural compound used in the clinic as an immunosuppressant for organ transplant patients and was first described as an immunosuppressive drug that blocked IL-2 induced proliferation. Rapamycin binds to cellular peptidyl propyl cis/trans isomerases, known as FK506-binding proteins (FKBPs)(456-461). Rapamycin binds to FKBP12 forming the active drug-protein complex necessary for the immunosuppressive effect of rapamycin in T cells (462-464). In mammalian cells mTOR phosphorylates and regulates two proteins, the ribosomal S6 Kinases that control ribosome biogenesis and 4E-BP1 (also known as PHAS-1) a repressor of translation initiation factor eIF4E. Non-phosphorylated 4E-BP1 binds and inhibits eIF4E. When 4E-BP1 is activated by mTOR/PI3K mediated phosphorylation it disassociates from eIF4E and allows it to bind other initiation complex proteins promoting translation initiation (465-467).

The 40s ribosomal S6 kinases (S6Ks) are the most well defined substrates of mTOR and were the first identified cellular targets for inhibition by rapamycin (456, 461, 468-472). The function of S6Ks was initially discovered by experiments showing that rapamycin suppressed the serum induced translational up regulation of a family of mRNAs containing a polypyrimidine tract at the 5' end (5'-terminal oligopyrimidine [5' TOP] mRNAs) (473-475). These mRNAs code for transcriptional apparatus such as ribosomal proteins and elongation factors (476) (reviewed in (477)). In mammals two S6K isoforms have been identified, S6K1 and S6K2. The role of S6K1 as a regulator of

cellular growth has been clearly illustrated by gene deletion studies in mice where the deletion of S6K1 is not lethal but results in mice with a 20% smaller body size at birth (478). S6K1-/-S6K2-/- mice are perinatally lethal (479). The regulation of S6K1 is complex involving multiple phosphorylation events (399, 480-482) and reviewed in (483). Crucial events include PDK1 mediated phosphorylation of threonine 229, which lies in the activation loop of the kinase domain (399, 484) and TOR phosphorylation of Thr229, Thr389 (485). The precise sequence of these phosphorylations is not known but the ability of TOR to phosphorylate S6Ks is regulated by cellular amino acid concentration (486-488). It is not known how mTOR senses amino acid levels but several models have been proposed: mTOR may sense charging or aminoacylated tRNA (486) or are regulated directly by amino acids, their metabolites or amino acid activated second messengers (489). Other key facts about the activation of S6Ks are that it requires activation of PI3K/PKB. Moreover, the effects of PKB on S6K are mediated by TSC2 (452, 490) and involve the GTPase Rheb. TSC2 acts as a GTPase-activating protein (GAP) towards Rheb and stimulates conversion of this GTPase from an active GTP-bound form to an inactive GDP-bound state. TSC2 inactivates Rheb, which results in the down regulation of TOR (491-494). PKB phosphorylation of TSC2 blocks its action as a Rheb GAP and promotes the accumulation of active GTP loaded Rheb which then activates mTOR.

1.8.1 Two mTOR pathways: Raptor and Rictor

The nutrient-sensitive mTOR complex required for downstream signalling is a complex of TOR and an adapter known as raptor although the mechanism is still

under debate (495, 496)(reviewed in (468)). GβL is thought to bind and stimulate the ability of mTOR to auto-phosphorylate (497-501). However, it has recently been shown that mTOR is also part of a distinct complex defined by the novel protein rictor (rapamycin-insensitive companion of TOR).

Rictor was identified through homology to yeast proteins known to be part of a rapamycin-insensitive TOR complex that does not contain raptor and signals in yeast to the actin cytoskeleton through Protein Kinase C 1 (PKC1) (502). Congruently, the rictor-containing mTOR complex contains GβL not raptor. This complex doesn't regulate S6K1 phosphorylation nor does it bind to FKBP12-rapamycin rather the rictor-mTOR complex modulates the phosphorylation of the actin cytoskeleton (503). For example HeLa cells deficient or knocked down for rictor display a perturbed morphology with actin formations differential to those seen in control cells (503). The rictor-mTOR complex is not sensitive to rapamycin but the PI3K inhibitor LY294002 seems to effect rictor-mTOR function suggesting this complex mediates functions assigned to PI3K.

The function of rictor-mTOR complexes in T cells is totally unexplored although recent data from David Sabatini's lab has shown that rictor-mTOR is necessary for the phosphorylation of Ser473 on Akt/PKB in an LY294002 and wortmannin sensitive manor (400), and facilitates PDK1 phosphorylation of Thr308. Hence γc cytokines may regulate PKB via the rictor-mTOR complex.

The importance of the raptor/TOR complex T cells can be inferred from the immunosuppressive effects of rapamycin which can inhibit the ability of IL-2 to regulate T cell size and cell cycle progression (357, 360, 389, 504). There are conflicting reports on how T cells respond to rapamycin. For example Breslin et

al suggest that IL-2 regulated expression of D type cyclins is sensitive to rapamycin and PI3K inhibitor LY294002 (352, 505). In contrast, Terada et al suggest that rapamycin down regulates S6K1 but does not inhibit T cell cycle progression in T cells already cycling as a result of IL-2 stimulation (506). It has also been reported that different γc cytokines show differential sensitivity to rapamycin e.g. IL-15 induced T cell proliferation is also more sensitive to inhibition of mTOR function by rapamycin than IL-2 responses (507).

One explanation for reported discrepancies in the sensitivity of T cell responses to rapamycin has recently been reported by Craig Thompson's lab who identified the Pim kinases as being able to substitute for rapamycin sensitive signalling pathways to control lymphocyte growth and survival. Pim kinases are a family of proto-oncogenes encoding a distinct class of serine/threonine kinases consisting of Pim1, Pim2 and Pim3.

It is known that IL-2 induces expression of Pim 1 (205). Mice deficient in Pim 1, 2 and 3 mice are viable and fertile but show a dramatic loss in body size and have severely impaired responses to growth factors in haematopoietic cell populations (508). Thus it is clear that Pim kinases are important to growth factor signalling. The relevance of Pim1 for γc signalling is demonstrated by studies of Pim1 transgenic mice (509-511). Where ectopic expression of Pim1 as a transgene can rescue the thymocyte developmental defects seen in IL-7-/- or γc-/- deficient mice (511).

Thompson and colleagues have recently probed cytokine signalling in Pim1/2 deficient mice and observed that T cell survival is rapamycin sensitive in their absence but rapamycin resistant when they are present. Pim kinases thus seem

to compensate for rapamycin independent regulation of T cell functions (512, 513).

1.8.2 Regulation of T cell growth

Analysis of the mechanism of action of rapamycin and work on Pim kinases has revealed the importance of changes in T cell metabolism for immune responses (434, 514-516). During an immune response, small metabolically inactive naïve T cells must rapidly increase basal glucose metabolism and rates of protein synthesis to satisfy the biosynthetic demands associated with execution of effector function (434, 517). Accordingly, the transcriptional response of effector T cells includes up regulation of genes encoding ribosomal components so that there is a coordinate and matched increase in cytokine gene expression and protein synthesis capacity (518). Reinforcement of the idea that immune responses are dependent on the ability of T cells to modulate their metabolism stems from work on the co-stimulatory molecule CD28 that regulates glucose metabolism during the primary response of T cells to antigen (437). It has also been recognised that the importance of Rel/NFκB transcription factors for T cell immune responses in part reflects their role in the regulation of T cell size (519).

The differentiation of antigen activated T cells to mature effectors takes several days and, as discussed, γc cytokines play a key role. These cytokines, presumably, need to maintain high rates of cell metabolism for a prolonged period to sustain the T cell differentiation process. Different γc cytokines all control distinct aspects of T cell differentiation, suggesting that they may differ in molecular responses induced within T cells (129, 133, 200, 227, 520-524).

However, analysis of cytokine signalling has focused on immediate biochemical and transcriptional programs where there is a commonality in the responses induced by γc cytokines and does not explain their unique functions (205, 206, 525).

One unexplored area of γc cytokine signal transduction is how cytokines compare in their effects on basic processes of cell growth. The question of how T cell cytokines regulate T cell growth is important because even if different cytokines can induce a common genetic program, differences in how they modify protein synthesis and cellular energy metabolism will influence how this genetic program is executed. Hence the term mitogen or growth factor, are frequently used interchangeably to describe γc cytokines but they are not synonymous. A growth factor is a molecule that regulates cell metabolism, protein synthesis and cell mass, whereas a mitogen controls cell division and proliferation. These are not the same processes nor are growth and cell division necessarily directly coordinated in eukaryotic cells (526-528). There is some information about how cytokines act as metabolic regulators for T cells; IL-7 regulates glucose metabolism in naïve T cells (435). Moreover, IL-2, the first cytokine shown to drive mitosis in activated T cells, was originally called T cell growth factor because of its ability to simultaneously induce cell cycle progression and induce an increase in cell size (529). As discussed, the mitogenic role of IL-2 can be supplanted by other γc cytokines (128) but IL-2 has essential non redundant functions *in vivo* for the efficient production of effector cytotoxic T cells, for sustaining T cell expansion and induction of tolerance (129-134).

The molecular basis for this unique signalling capacity of IL-2 is not known but one possibility is that it is due to the actions of IL-2 as a growth factor rather than its role as a mitogen. Support for this hypothesis stems from reports that other cytokines known to control T cell proliferation may not be as potent growth factors as IL-2. For example, IL-7 is very important for the survival of naïve CD8+ T cells but these survive as small cells not large lymphoblasts. Moreover, antigen primed T lymphoblasts cultured in IL-7 rapidly regain a small cell phenotype compared to large IL-2 blasts (229).

Similarly, IL-15 is equal to IL-2 in it's capacity to promote mitogenesis and clonal expansion of antigen primed CD8+ effector T cells *in vitro* but IL-15 maintained CD8+ T cells are decreased in cell size compared to IL-2 lymphoblasts (48, 51, 229). IL-15 induced T cell proliferation is also more sensitive to inhibition of mTOR function by rapamycin than IL-2 responses (507); a further indication that these cytokines may not be equivalent in terms of protein synthesis. The possibility that γc cytokines are not equivalent growth factors for T cells affords the insight that understanding the biochemistry of cell growth control in lymphocytes will be the key to understanding the unique actions of different members of the γc cytokine family.

1.9 Thesis aims

The object of the present thesis was to explore the ability of γc cytokines to regulate T cell growth. The main focus of the present study was to compare the actions of IL-2 and IL-15 on protein synthesis and cell growth of antigen activated CD8+ T cells. The choice of this model is based on previous observations that IL-2 and IL-15 are equivalent mitogens for these cells *in vitro*

but differentially regulate T cell size: antigen primed CD8+ T cells cultured in the presence of IL-2 differentiate into large cytotoxic effector T cells whereas T cells maintained with IL-15 do not and rather develop into small cells with a phenotype reminiscent of CD8+ memory T cells (48, 51). The present study explores the signalling pathways used by IL-2 and IL-15 to control T cell protein synthesis and reveals the basis for the differential size response of T cells to these cytokines.

Chapter 2

Methods and Materials

2.1 P14 LCMV TCR Transgenic Mice

P14 TCR transgenic Mice (530) were bred and maintained in the Cancer Research UK Biological Resources Unit and the Wellcome Trust Biocentre, University of Dundee Transgenic animal unit in compliance with UK Home Office Animals (Scientific Procedures) Act 1986 guidelines.

2.1.1 LCMV specific peptide

The P14 TCR recognises LCMV gp(33–41) (KAVYNFATM) in the context of H-2D^b (531). This peptide was synthesised and purified in the Cancer Research UK protein production laboratory.

2.1.2 Mice typing by flow cytometry

Tail blood from P14 TCR transgenic mice was screened by flow cytometry for T cell expression of TCR Vβ8, Vα2, T cell specific CD3 antigen and co-receptors CD4 and CD8. 50μl of blood was transferred into FACS tubes and the red blood cells lysed with 300μl of ACK lysis buffer (see cell culture) for 10 min at room temperature or until the solution is clear. The cells were washed in FACS buffer (see flow cytometry) and stained using saturating concentrations of anti-Vβ8,

anti-Vα2, anti-CD3, anti-CD4 and anti-CD8 antibodies. Expression of the transgenic TCR Vβ8:Vα2 and CD4:CD8 T cell ratios were determined by CellQuest software (Beckton Dickinson).

2.2 Cell culture and organ preparation

2.2.1 Isolation and Preparation of Lymphoid Organs

Spleens and Lymph nodes were obtained by dissection from 1-3 month old mice. Tissue was disaggregated using a 2μM mesh cell strainer to obtain a single cell suspension. Splenocytes were treated with ACK lysis buffer for erythrocyte lysis and washed thoroughly. Cell numbers were adjusted according to subsequent application (1-10 x 10^6 cells/mL).

2.2.2 *In vitro* T cell activation and cell culture

Murine T cell blasts were made from splenocyte and lymph node cell suspensions, adjusted to 5x10^6 cells/ml, and cultured in RPMI/DMEM, 10%FBS (heat inactivated), βME 50μM and penicillin/streptomycin. The cells were stimulated with soluble LCMV specific peptide gp33-41 (1uM) or 2C11 (1-5μg/ml) antibody (Cancer Research UK hybridoma unit) for 48hours, washed and resupsened in 20ng/mL of cytokine (IL-2, IL-15, IL-4, and IL-7), unless indicated differently, or medium only as a control. Where indicated the cells were incubated in specified concentrations of a kinase inhibitor.
To inhibit PI3K LY294002 (Promega) or wortmannin (Sigma) was added to cell cultures at a final concentration of 10μM and 100nM respectively.

To inhibit mTOR rapamycin was added to cell cultures at a final concentration of 20nM.

2.2.3 Live cell imaging for microscopy

T cell blasts were adjusted to $4x10^6$ cells/ml in RPMI-1640, 10% FBS, 50μM 2ME, penicillin/streptomycin and added to a glass bottomed Matex dish (Matex) previously coated with murine ICAM-1 5μg/ml in PBS, for one hour at 37°C. Cells were left to adhere in serum free medium for 30 min at 37°C and imaged in RPMI-1640, 10% FBS, 50μM 2ME, and penicillin/streptomycin.
Images were taken in a 37°C humidified environmental chamber provided with 5% CO_2 in balanced air. Specimens were analysed with a Carl Zeiss LSM510 confocal imaging system (Zeiss, Jena, Germany) equipped with a 63x NA 1.4 plan apochromat lens.
Recombinant Mouse ICAM-1/Fc Chimera (R&D Systems)

2.2.4 Cell Culture Reagents and Solutions

Reagents

Recombinant human IL-2 (Proleukin, Chiron.B.V, Netherlands)

Recombinant Human IL-15 and IL-7, mouse IL-4 (Peprotech)

Recombinant Mouse ICAM-1/Fc Chimera (R&D Systems)

2-Betamercaptoethanol (β-ME) (Sigma)

LY294002 [2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one], use at 10μM

Wortmannin use at 100nM (Calbiochem)

Rapamycin 20mM (Calbiochem)

DMSO sterile (sigma)

Roswell Park Memorial Institute-1640 (RPMI 1640) (GIBCO)

Dulbecco's modified Eagle's medium (DMEM) (GIBCO)

Foetal Bovine Serum FBS (GIBCO) EU approved

Penicillin/Streptomycin (Pen/Strep) (GIBCO)

Media and Solutions

RPMI-1640 + L-Glutamine (GIBCO) 10% FBS (heat Inactivated), 50μM 2ME, Pen/Strep (GIBCO)

DMEM + L-Glutamine (GIBCO) 10% FBS (heat Inactivated), 50μM 2ME, Pen/Strep (GIBCO)

ACK lysis buffer: 4.145g NH_4Cl, 0.5g $KHCO_3$ and 0.018g EDTA pH 7.8

2.3 Flow Cytometry

2.3.1 Live cell staining

Antibodies were obtained conjugated to fluorescent isothiocyanate (FITC), phycoerythrin (PE) and Allophycocyanin (APC). Prior to staining, Fc Receptors were blocked with an anti-FcγRII blocking monoclonal antibody. Cells were then stained at 1 $x10^6$ cells per sample with previously titrated concentrations of antibody at 4°C for 20 minutes or 1hour in FACS buffer. Cells were washed and resuspended in FACS buffer prior to acquisition on a FACS Calibur (Becton Dickinson, San Jose, CA). Events were collected, stored ungated and data analysed with CellQuest (Becton Dickinson) software. Live cells were gated according to their forward light scatter (FSC) and side light scatter (SSC) profiles.

2.3.2 Live cell cycle analysis

Cellular DNA content was assayed on live cells using 7 amino actinomycin D (7AAD) staining. Cells (1 x 10^6 cells) were incubated for 1 hr at 37°C in 7AAD staining solution. Data was analyzed by FACS using CellQuest software and doublet discrimination.

7AAD staining solution: 20 g/ml 7AAD, 25 g/ml Rnase and 0.03% saponin in PBS/20 mM HEPES (pH 7.4)/2% heat-inactivated FBS

Cellular DNA content was assayed using the dye Ho33342 (Hoescht). 20ug/mL of Hoescht dye was added to 10^6 cells and incubated at 37°C for 50 minutes before being analysed by flow cytometry using the LSR (Becton Dickinson) flow cytometer UV laser.

2.3.3 Phospho-S6 ribosomal protein intracellular staining

After indicated treatment at 37°C, T cells were washed and fixed in 0.5% PFA for 15 minutes at 37°C, washed twice in PBS and followed by a precise10 minute incubation in 90% methanol at -20°C. Cells were washed twice in PBS and blocked in BSA buffer for 10 minutes at room temperature. Cells were incubated with 1:100 dilution of primary anti-phospho-S6 (cat # 2211; Cell Signalling Technologies) in BSA buffer for 30 minutes at room temperature, washed once with BSA buffer and incubated with 1μg of FITC conjugated donkey anti-rabbit IgG (cat# 711-096-152; Jackson ImmunoResearch) for 30 minutes at room temperature in the dark. Samples were washed in BSA buffer and analysed on a FACSCalibur (Becton Dickinson).

Pharmacological stimulation for 30 minutes at 37°C with 2μg/mL of the phorbol ester, phorbol 12.13 dibutyrate (PdBu), was used to stimulate all available S6 protein in the cell and served as a positive control. Pharmacological inhibitor treatments were undertaken for 30 minutes at 37°C before fixation. (Rapamycin 20nM, LY294002 10μM, Wortmannin 100nM)

anti-phospho-S6 (cat # 2211; Cell Signalling Technologies)

2.3.4 Flow Cytometry Reagents and Solutions

Reagents

7aad (7 amino actinomycin D) (Sigma)

Hoechst 33342 10mg/ml, Molecular probes

Methanol

Paraformaldehyde (Sigma)

PBS tablets (Sigma)

BSA (Sigma)

Solutions

FACS Buffer: DMEM/RPMI minus Phenol Red, 25mM Hepes (pH7.4 with HCl), 1% FBS

0.5% PFA: 0.05g Paraformaldehyde, 10mL PBS (tablets, Sigma)

BSA Buffer: 0.5% BSA, PBS (tablets, Sigma)

Antibodies

Directly conjugated: R-Phycoerythrin (RT-PE); Fluorescein Isothiocyanate (FITC); Allophycocyanin (APC) Pharmingen.

Vβ8	Clone F23.1, isotype: Mouse IgG2a, κ
CD3ε	Clone 145-2C11, isotype: Armenian Hamster IgG1, κ.
CD4 (L3T4)	Clone RM4-5, isotype: Rat IgG2a, κ, Clone GK1.5, isotype: Rat IgG2b, κ.
CD8α (Ly-2)	Clone 53-6.7, isotype: Rat IgG2a, κ.
CD25 (IL-2 Receptor α chain, p55)	Clone 3C7, isotype: Rat (lewis) IgG2b,κ
CD44 (Pgp-1, Ly-24)	Clone IM7, isotype: Rat IgG2b,κ
CD45R/B220	Clone RA3-6B2, isotype: Rat IgG2a,κ
CD62L (L-selectin, LECAM-1, Ly-22)	Clone MEL-14, isotype: Rat (Fischer) IgG2a,κ
CD71 (Transferrin Receptor)	Clone C2 (C2F2), isotype: Rat IgG1,κ
CD90.2 (**Thy1.2**)	Clone 53-2.1, isotype: Rat IgG2a, κ
4G3 Common γ chain (IL-2R, IL-4R, IL-7R Shared γ subunit)	Clone 4G3, isotype
Vα2	Clone B20.1, isotype: Rat IgG2a, λ

Directly conjugated: Tri-colour (TC) Caltag

CD4	Clone RM4-5, isotype: Rat IgG2a

CD8α	Clone 5H10, isotype: Rat IgG2b

Purified Antibodies

CD16/CD32 (FcγIII/II Receptor)	Monoclonal Antibody (Mouse BD **Fc Block**) clone: 2.4G2, isotype IgG2b,κ
CD98 (4F2)	Clone H202-141, isotype: Rat IgG2a, κ
IL-15Rα	isotype: Goat IgG1 (R & D Systems)
Anti-phospho-S6	Rabbit antibody (cat # 2211; Cell Signalling Technologies)

FITC conjugated Secondary Antibodies (Jackson ImmunoResearch)

AffiniPure F(ab')$_2$ Fragment Donkey:	Anti-Rabbit IgG (H+L)
AffiniPure Mouse:	Anti-Goat IgG (H+L)
AffiniPure Goat:	Anti-Rat IgG (H+L)

2.4 Proliferation assay

Antigen activated P14 LCMV CD8+ T cells were re-suspended in RPMI1640/10% FBS/50μM βME, and seeded in triplicate at 5 x 10^4-1 x 10^5 cells/well in 96 well-flat bottom microtitre plates. Cells were cultured with medium alone or different concentrations of cytokines. Cells were then cultured at 37°C in a 5% CO_2 humidified incubator for the indicated time. Prior to harvesting 1μCi of tritiated (^{3}H) thymidine (Amersham, UK) was added to each well and cells incubated for a further 2 hours. Cells were harvested using vacuum aspiration onto glass matrix filters. Incorporated radioactivity was quantified using a β-microplate scintillation counter (532).

[methyl-^{3} H] Thymidine (Amersham Biosciences)

2.5 Amino acid uptake into cells

The rate of amino acid uptake into antigen activated P14 LCMV CD8+ T cells in response to different concentrations of cytokine or medium alone was assayed by re-suspending antigen activated CD8+ T cells in RPMI 1640/10% FBS/50μM βME, and seeding in triplicate at 1 x 10^5 cells/well in 96 well-flat bottom microtitre plates. Cells were then cultured at 37°C in a 5% CO_2 humidified incubator for the indicated time. Tritiated (^{3}H) amino acid mix (Amersham, UK), 2μCi/well, was added to the plates for 6 hours prior to harvesting by vacuum aspiration on glass matrix filters. Incorporated radioactivity was quantified using a β-microplate scintillation counter.

[^{3} H] Amino Acid mix, Amersham Biosciences

2.6 Amino acid incorporation into cellular protein

To determine amino acid incorporation into cellular protein, antigen stimulated P14 LCMV CD8+ T cells were cultured in cytokine or medium alone at 5 x 10^6 cells/mL and cells collected at indicated time points. Cells were pulsed for 12 hours, prior to harvest, with 5μCi/mL tritiated (^{3}H) amino acid mix (Amersham, UK). Cell pellets (5 x 10^6 cell/pellet) were harvested in triplicate and lysed in 1mL of lysis buffer (see western blotting) for 20 min at 4 °C. The resulting extract was centrifuged at 15,000g for 30 minutes at 4 °C and resultant supernatant transferred to fresh eppendorff tubes and protein was precipitated by adding an equal volume of 70% acetone and incubating for 1hour at 20 °C. The solution was then centrifuged at 15,000g for 30 minutes supernatant removed and pellet dissolved in water before incorporated radioactivity was quantified on a β scintillation counter.

2.7 Protein Content

Cell pellets containing 1 x 10^6 cells were lysed at 4 °C in 1mL of lysis buffer (see western blotting) and cell debris removed by centrifugation at 15,000g for 30 minutes and aliqots taken for analysis of protein concentration. Protein content was quantified using the BIORad protein assay dye reagent, referenced against a standard curve of BSA. All samples were analysed by spectrophotometry (Jenway 6405 UV/Vis) at 595nm.

2.8 Cytotoxic assay

The JAM test was performed to evaluate the antigen specificity of LCMV TCR positive effector and central memory CD8+ T cells (533). Peptide-pulsed EL4 thymoma cells were used as target/stimulator cells. Antigen loaded and [H^3] thymidine-labelled EL4 cells were generated by pre-incubation with 5 μCi/mL [H^3] Thymidine and 1-10μM of peptide overnight at 37°C. Peptides used in this assay included the LCMV gp(33–41) (KAVYNFATM) in the context of H-$2D^b$ (530), recognising the P14 TCR, and the control peptide, influenza np(60–68) (ASNENMDAM) in the context of H-$2D^b$ (534), specific for the F5 TCR. These peptides were synthesised and purified in the Cancer Research UK Protein Production Laboratory.

The T cell blasts of interest were added to a 96 flat-bottomed plate and diluted in a series from 1 x 10^6/well to 1 x 10^4/well and the labelled 'target' EL4 cells were added to the plate (1 x 10^4 cells/ well or 1 x 10^5 cells/mL). Target cells and effector cells were co-incubated for 2 h at 37°C, plates were harvested by vacuum aspiration on glass matrix filters. Incorporated radioactivity was quantified using a β-microplate scintillation counter (LKB Wallac 1205 Betaplate® liquid Scintilation counter).

The percent specific lysis of target cells by CTL was calculated, E = Experimentally retained DNA in the presence of killers (in CPM), S = retained DNA in the absence of killers (spontaneous) incorporated, so % specific killing = [(S-E)/ S] X100.

2.9 Western Blotting

Cell pellets containing 10 x 10^6 cells were harvested and lysed in 1mL lysis buffer for 30 min at 4°C, centrifuged at 15,000rpm for 30 min at 4 °C and transferred to clean eppendorff tubes. [Protein content was determined at this stage using the BIORad protein assay dye reagent, referenced against a standard curve of BSA]. Resultant supernatant was incubated with equal volumes of -20°C 70% acetone, to precipitate protein, for 1 hour at -20 °C and centrifuged at 15,000g for 30 minutes at 4 °C. Pelleted protein samples were air dried, boiled at 100°C in reducing sample buffer for 5 minutes and shaken overnight to dissolve protein. All samples were boiled for 5 minutes prior to being separated on 7.5% SDS-PAGE gels at a concentration of 100μg/sample for 12 hours at 50 volts. Protein was transferred by electro-blotting onto polyvinylidene diflouride (PVDF) membranes over 4 hours at 0.4mA (70volts) in 10mM CAPS transfer buffer at pH 11. Efficiency of protein transfer was checked using ponceau S staining of the membrane and washed off with PBS-Tween20. Membranes were then blocked in 4% milk solution (dried fat free milk powder dissolved in PBS-Tween20) and incubated overnight at 4°C with primary antisera diluted in BSA blotting solution. Membranes were washed (3x 10 minutes) in PBS-Tween20 and incubated with HRP linked secondary antibody diluted in milk blotting solution at room temperature for one hour. The membrane was washed (3x 10 minutes) in PBS-Tween20 and protein visualised using chemiluminessence detection (ECL, Amersham).

Subsequent primary antibodies were applied to membranes after stripping in buffer at 56°C for 30 minutes, washing in PBS-Tween20 and blocking in 4% milk solution.

2.9.1 Western Blotting Solutions and Reagents

Solutions

Stock 2x cell lysis buffer: 100mM HEPES @ pH 7.4, 300mM NaCl, 20mM NaF, 20nM Iodoacetamide (IAA)

Working cell lysis buffer; 5mL 2x stock lysis buffer, 10% NP40, 100mM PMSF in ethanol, 1 complete protease inhibitor cocktail tablet (Sigma), add water to 10mL volume.

i.e. (Final 10mL solution; 50mM HEPES at pH 7.4, 150mM NaCl, 10mM NaF, 10mM iodoacetamide (IAA), 1% NP-40, 1mM phenyl methyl sulphonyl fluoride (PMSF), 1 protease inhibitor cocktail tablet, EDTA-free (Pancreas extract 0.02, chymotrypsin 0.02, thermolysin 0.02, trypsin 0.02, papain 0.33: mg/ml, Roche)).

Reducing sample buffer: (3x) 30% (w/v) glycerol, 187.5mM Tris at pH 6.8, 10% (v/v) 2-Betamercaptoethanol (β-ME), 9% (w/v) SDS

Transfer buffer: 10mM CAPS buffer at pH 11.

Running buffer: 25mM Tris pH 8.3, 190mM glycine, 3.5mM SDS.

Stripping buffer: 36.5mL H_2O, 10mL 10% SDS, 3.125mLTris 1M pH 6.8, 0.347mL β-ME.

PBS-Tween: PBS, 0.05% Tween20

Membrane blocking: 4% fat free milk powder (Marvel) dissolved in PBS, 0.05% Tween20 (PBS-T)

Primary Antibody BSA blotting: 1% BSA, 0.05% azide in PBS-Tween20

Secondary detection antibody milk blotting: 4% fat free milk powder (Marvel) dissolved in PBS-Tween20

SDS-page acrylamide protein gels

7.5%: 11.2mL 1M Tris at pH8.8, 11.2mL water, 7.5mL Protogel, 300μL 20% (w/v) SDS; 100μL 10% (w/v) ammonium persulphate (APS), 20μL tetramethylethylenediamine (TEMED) for cross-linking acrylamide (30mL total volume)

4% stacking gel; 2.5mL1M Tris at pH6.8, 13.3mL water, 4mL Protogel, 200μL 20% (w/v) SDS; 100μL 10% (w/v) ammonium persulphate (APS), 20μL tetramethylethylenediamine (TEMED) for cross-linking acrylamide (10mL total volume). Cast over the resolving gel to aid sample resolution.

Reagents

Ponceau S (Sigma)

BioRad Dye reagent concentrate, BioRad.

Rainbow coloured protein molecular weight markers, Amersham Biosciences

Protease Inhibitor Cocktail Tablets Complete, Mini, EDTA-free (Pancreas extract, Chymotrypsin, Thermolysin (Metalloprotease) and Trypsin 0.02mg/mL, Papain 0.33mg/mL), Roche

ECL (Pharmingen)

Temed (tetramethylethylenediamine) (Sigma)

Ammonium persulfate (Aps) (Sigma)

Azide (Sigma)

2-Betamercaptoethanol (β-ME)

SDS (Sigma)

TRIS-HCl (Sigma)

Polyvinylidene diflouride (PVDF) membranes. (immobilon-P, Millipore)

3mm blotting paper

Antibodies

Primary antibodies

2d4-perf	anti-Perforin mouse antibody (Diluted 1:1000) (A gift from Gillian Griffiths (535))
U5	monoclonal rabbit antibody against the BCR domain of p85α (536) 1.7ug/mL

Peroxidase Conjugated Secondary Antibodies

All were diluted 1:5000

Anti-Rabbit HRP	Amersham biotech
Anti-Mouse HRP	Amersham biotech

Chapter 3

IL-2 and IL-15 differentially control T cell differentiation *in vitro*.

3.1 Introduction

The initial aim of this thesis was to develop an *in vitro* model to compare the action of IL-2 and IL-15. These cytokines activate cells via a receptor that shares a common γ chain (γc, IL2Rγc, CD132), the IL-2Rβ chain (CD122) but has unique α chain subunits (IL-2Rα and IL-15Rα). IL-2 and IL-15 are known to initially induce common transcriptional programs (205) yet ultimately induce different biological outcomes *in vivo*. To fully understand these differences, extensive analysis of how these two cytokines signal in T cells is needed. It is very difficult to isolate sufficient primary activated T cells *ex vivo* for biochemical analysis and accordingly an *in vitro* model in needed to generate sufficient numbers of pure T cells for functional studies. In this respect, it has been known for more than 20 years that antigen activated T cells can be clonally expanded *in vitro* in the presence of IL-2 to generate cytotoxic effector T cells. More recently, the laboratory of Von Andrian described a similar *in vitro* model for the generation of effector memory-like CD8+ T cells (48, 49). This model uses a transgenic (Tg) mouse P14 (specific for the lymphocytic choriomeningitis virus glycoprotein peptide gp(33-41)). This protocol involves antigen activation of

mice splenocytes *in vitro* to generate a large population of antigen activated CD8+ T cells. These cells were then cultured in IL-2 or IL-15. The antigen activated T cells cultured in IL-2 differentiated into classical effector CTL. In contrast T cells cultured in IL-15 differentiate to cells that resemble memory T cells in that they are small and very inefficient cytotoxic effectors. However, these antigen activated T cells cultured *in vitro* with IL-15 could mediate rapid recall responses to antigen *in vitro* and *in vivo* e.g. the cells could survive for 10 weeks in a host after adoptive transfer and were capable of mounting a secondary immune response in response to antigen re-challenge that was as potent as an *in vivo* stimulated endogenous memory response (48). One other way in which the *in vitro* IL-15 cultured T cells resemble memory T cells is in their chemotactic and homing responses. Von Adrian's lab initially analysed antigen activated T cells cultured in IL-15 or IL-2 *in vitro* transwell assays. These data showed antigen activated cells cultured in IL-2 and IL-15 were able to migrate toward inflammatory cytokines *in vitro*, however cells cultured in IL-15 were more efficient at homing towards lymph node tropic chemokines suggesting that both cells cultured with IL-2 or IL-15 were able to home to sites of infection, but only cells cultured in IL-15 were responsive to lymph node homing chemokines (48). This *in vitro* data was supported by a set of *in vivo* adoptive transfer experiments where they found that naïve, cells cultured in IL-15 and, to a lesser extent, cells cultured in IL-2 homed to the T cells areas of the spleen. However, only naïve and cell cultured in IL-15 homed to the lymph nodes and Peyer's patches (51, 537). Again, using intravital microscopy, showed antigen activated T cells cultured in IL-15 not IL-2 could roll and arrest in high endothelial venules (HEVs) (51). Also in a peritonitis inflammation model

they were able to show that both cells cultured in IL-2 and IL-15 were efficient at homing to sites of inflammation when compared to naïve cells, although cells cultured in IL-2 were significantly more efficient (51). Thus, *in vitro* and *in vivo* migration data confirmed that these *in vitro* generated antigen activated T cells cultured in IL-15 home avidly to lymphoid organs and moderately to sites of inflammation, reminiscent of central memory T cells, and cells cultured in IL-2 accumulated in inflamed tissue but excluded from most lymphoid organs, typical of effector CTL (51). We considered that the system described by Von Andrian would be good for the comparisons of IL-2 and IL-15 signal transduction. It could also allow for the generation of unlimited numbers of memory CD8+ T cells and thus possibly open up an avenue for biochemical analysis of cytokine signalling in memory verse naïve T cells. Accordingly an initial objective of the thesis was to evaluate the potential of antigen primed T cells derived from the P14 LCMV TCR transgenic model to be used as a system for the comparison of IL-2 and IL-15 signal transduction.

3.2 Results

3.2.1 IL-2 and IL-15 are potent T cell mitogens but have a differential effect on T morphology and function.

In the present study we used primary CD8+ T cells derived from the P14 LCMV TCR transgenic model where peripheral CD8+ T cells express a Vα2/Vβ8.1 TCR specific for the immunodominant lymphocytic choriomeningitis virus (LCMV) glycoprotein peptide, gp33-41 (KAVYNFATM), presented in the context of H-2D^{b} class I MHC molecule (538). The efficient positive selection of the P14 TCR cause P14 mice to have an abnormal ratio of CD4:CD8 T cells were CD8 T

cells out number CD4 by 4:1, differing from a normal T cell distribution of 1:2 (CD8:CD4).

Lymph node and spleen were taken from the P14 mice, mashed through a cell strainer, re-suspended vigorously for 30 seconds in ACK buffer, to lyse the majority of unwanted red blood cells, and washed twice in RPMI supplemented with 10% FBS, 2ME and penicillin/streptomycin. The cell suspension (comprising of T cells and antigen presenting cells) was fixed to 5 x 10^6 cells/mL and treated with 1μM of the LCMV gp33-41 specific peptide (hereafter referred to as gp33 peptide) for two days, in the absence of exogenous cytokine. This initial stimulation specifically activates the high number of CD8+ T cells in the population which express the LCMV Vα2/Vβ8.1 TCR and allowed us to generate a large pool of activated CD8+ T lymphoblasts. After the two-day activation these cells were washed free of antigen and cultured in medium alone or in the presence of a cytokine as indicated. The data in **Fig 3.2.1a** compares the effect of different concentrations of IL-2 or IL-15 on the ability of these antigen activated T cells to incorporate tritiated thymidine into newly synthesised DNA. Thymidine incorporation measures rates of DNA synthesis and hence allows a comparison of the frequency of cells in the S phase of the cell cycle. The data in **Fig 3.2.1a** show that both IL-2 and IL-15 are comparable in their ability to initiate DNA synthesis in antigen primed CD8+ T cells. A true comprehensive measure of proliferation is to count cell number increases over time in culture. The data in **Fig 3.2.1b** show cell counts taken over 10 days in culture with 20ng/mL of IL-2 or IL-15. A parallel cell count of the same activated T cells cultured without exogenous growth factors was the medium only control. Antigen activated T cells cultured without exogenous growth factors (medium only control) do not

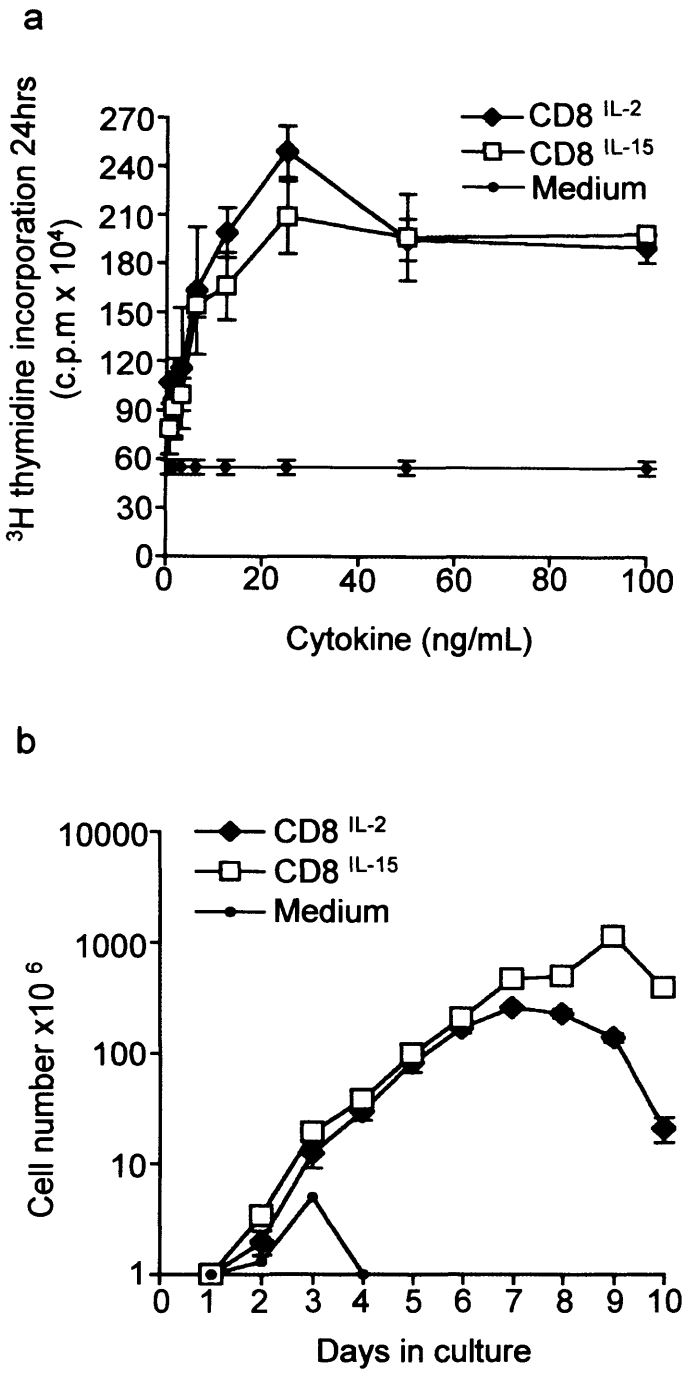
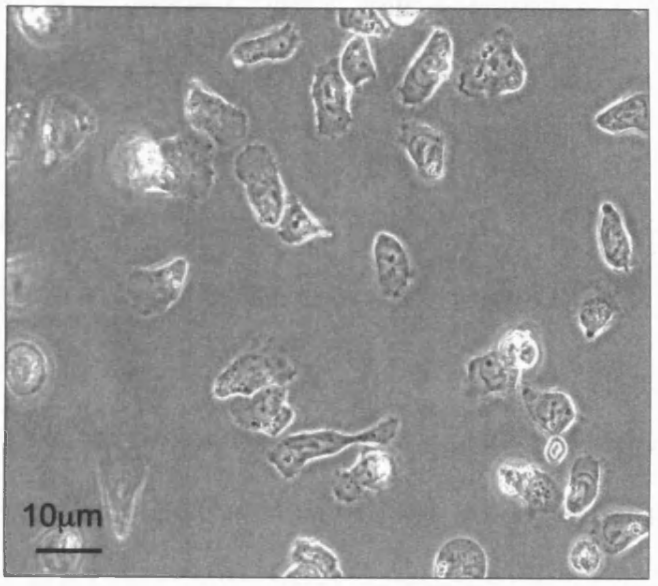


Figure 3.2.1: IL-2 and IL-15 are equivalent mitogens for antigen primed CD8+ T cells. a) Data show representative graphs of tritiated (^{3}H) thymidine incorporation into 2 day antigen activated P14 LCMV CD8+ T cells cultured with indicated concentration of IL-2 ($CD8^{IL-2}$), IL-15 ($CD8^{IL-15}$) or in medium alone for 24 hours. b) Cell numbers of antigen activated CD8+ T proliferating exponentially with 20ng/mL IL-2 ($CD8^{IL-2}$), IL-15 ($CD8^{IL-15}$) or medium only. These experiments were repeated 10-15 times.

proliferate and become apoptotic over 48-96hours and die. However, when antigen activated CD8+ T cells are cultured in the presence of mitogens IL-2 and IL-15 it is obvious that both cytokines have a potent ability to drive mitosis, as indicated by a rapid phase of clonal expansion resulting in an approximate 200-400 fold increase in cell number over a period of 6 days (**Fig 3.2.1b**). At later time points in the growth curve, day 6 in culture and onwards, the responses to the two cytokines diverge. T cells maintained in IL-2 stop proliferating and decline in number, due to an increase in the rate of apoptosis versus cell division. Although, in contrast, T cells maintained for a similar time in IL-15 no longer proliferate but support cell numbers in culture and are able to survive for prolonged periods of time (**Fig 3.2.1b**).

It has been described that antigen activated T cells cultured in the presence of IL-2 differentiate to effector cytotoxic T cells whereas cells cultured in IL-15 resemble central memory T cells (48, 49). The morphology of effector cells verses memory T cells is quite different. Accordingly, we have compared the morphology and phenotype of the antigen activated CD8+ T cells cultured in IL-2 ($CD8^{IL-2}$) or IL-15 ($CD8^{IL-15}$). In **Fig 3.2.2** the cell morphology of antigen activated CD8+ T cells cultured in IL-2 or IL-15 was visualised using live cell imaging with low light video microscopy. Activated CD8+ T cells cultured in the presence of 20ng/mL of IL-2 or IL-15 for 5 days were allowed to spread and migrate over glass bottomed matex dishes, coated with 5ng/mL recombinant mouse ICAM-1 for 1 hour at 37°C, in a humidified chamber for 30 minutes. The data show representative still images of these cells and reveal that antigen

CD8 $^{IL-2}$ T cells



CD8 $^{IL-15}$ T cells

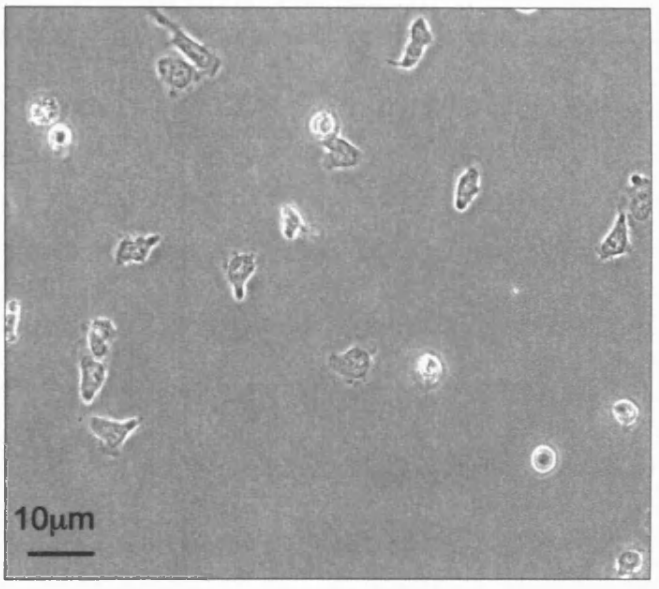


Figure 3.2.2: Antigen primed T cells cultured in IL-2 are bigger than T cells cultured in IL-15. Live cell images of antigen primed P14 CD8+ T cells cultured for 5 days in either 20ng/mL IL-2 ($CD8^{IL-2}$) or IL-15 ($CD8^{IL-15}$). Cells were allowed to settle on glass coated with 5ng/mL of recombinant mouse ICAM-1.

activated CD8+ T cells cultured in IL-2 ($CD8^{IL-2}$) appear larger and more granular than cells cultured in IL-15 ($CD8^{IL-15}$) (**Fig 3.2.2**).

The phenotype of CD8 $^{IL-2}$ verses CD8 $^{IL-15}$ cells was assessed by also flow cytometric analysis of cell surface markers commonly used to distinguish effector and memory CD8+ T cells.

Data in Fig 3.2.3 show $CD8^{IL-2}$ cells were $CD25^{High}$, $CD44^{High}$ and $CD62L^{Low}$, where cells maintained in IL-15 for the same time were $CD25^{Low}$, $CD44^{High}$ and $CD62L^{High}$.

Activated CD8+ T cells are defined by their role as effector or cytotoxic killer T cells. Analysis of cytotoxic activity associated with the two phenotypically distinct populations $CD8^{IL-2}$ and $CD8^{IL-15}$ was undertaken as it has been previously noted that effector memory CD8+ T cells ($CD8^{IL-15}$) loose their immediate killing capacity (41). Moreover, Von Andrian and colleges described $CD8^{IL-2}$ cells as having high cytotoxic activity and $CD8^{IL-15}$ cells as having very little cytotoxic potential (48).

One important molecule in cytotoxic T cells is the pore forming protein perforin. The data in **Fig 3.2.4a** shows western blot analysis of perforin expression in $CD8^{IL-2}$ verses $CD8^{IL-15}$ cells. The data show that $CD8^{IL-2}$ cells have high levels of perforin whereas CD8 $^{IL-15}$ cells have almost undetectable levels of perforin. These data are consistent with the described inability of IL-15 to support the differentiation of effector CTL. In additional experiments the cytotoxic potential of T cells cultured in IL-2 or IL-15 was compared using a method whereby the ability of T cells to kill a specific number of target cells was measured by the release of a radiolabel from a target cell indicating T cell lysis or death (533).

Target cells used were a thymoma cell line, EL4, which can present antigens in the context

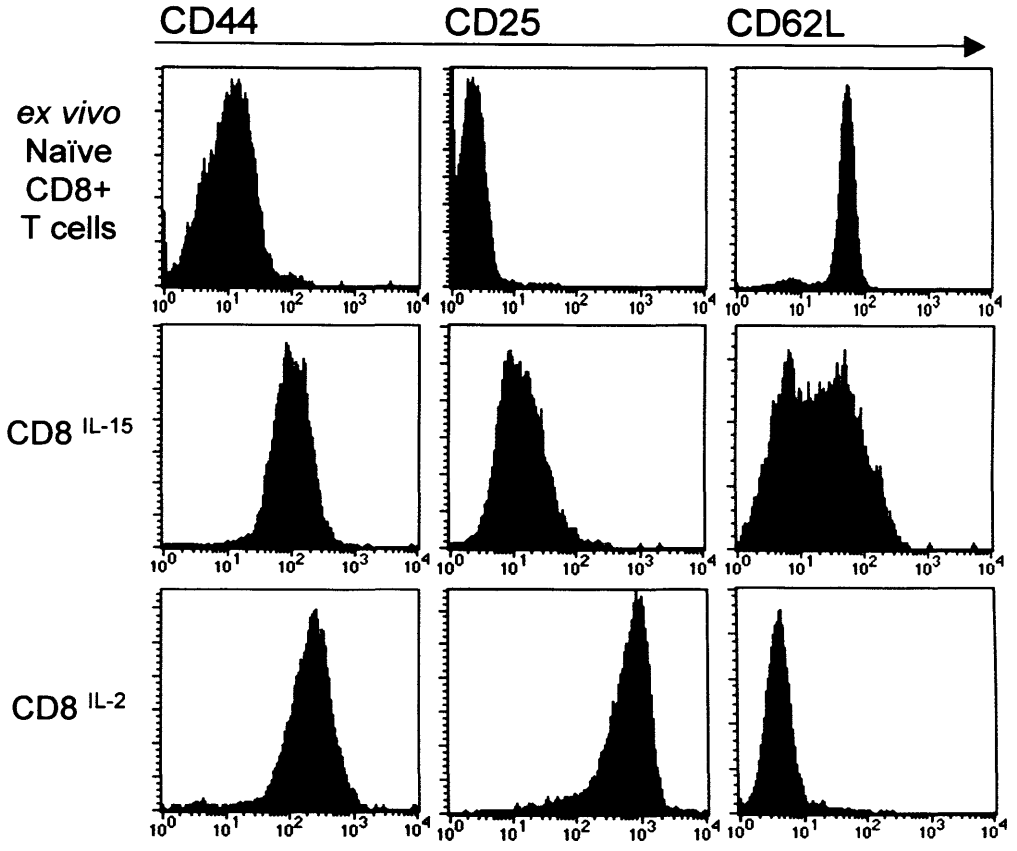


Figure 3.2.3: Antigen primed T cells cultured in IL-2 or IL-15 for 5 days express different surface markers when compared to *ex vivo* naïve CD8+ T cells.
Representative FACS histograms indicating surface levels of markers: CD44, CD25 (IL-2Rα chain) and CD62L (L-Selectin), on 2 day gp33 LCMV antigen primed P14 CD8+ T cells cultured for 5 days in either 20ng/mL IL-2 ($CD8^{IL-2}$) or 20ng/mL IL-15 ($CD8^{IL-15}$). *Ex vivo* CD8+ T cells were isolated by flow cytometric cell sorting. Staining profiles repeated over 10 times.

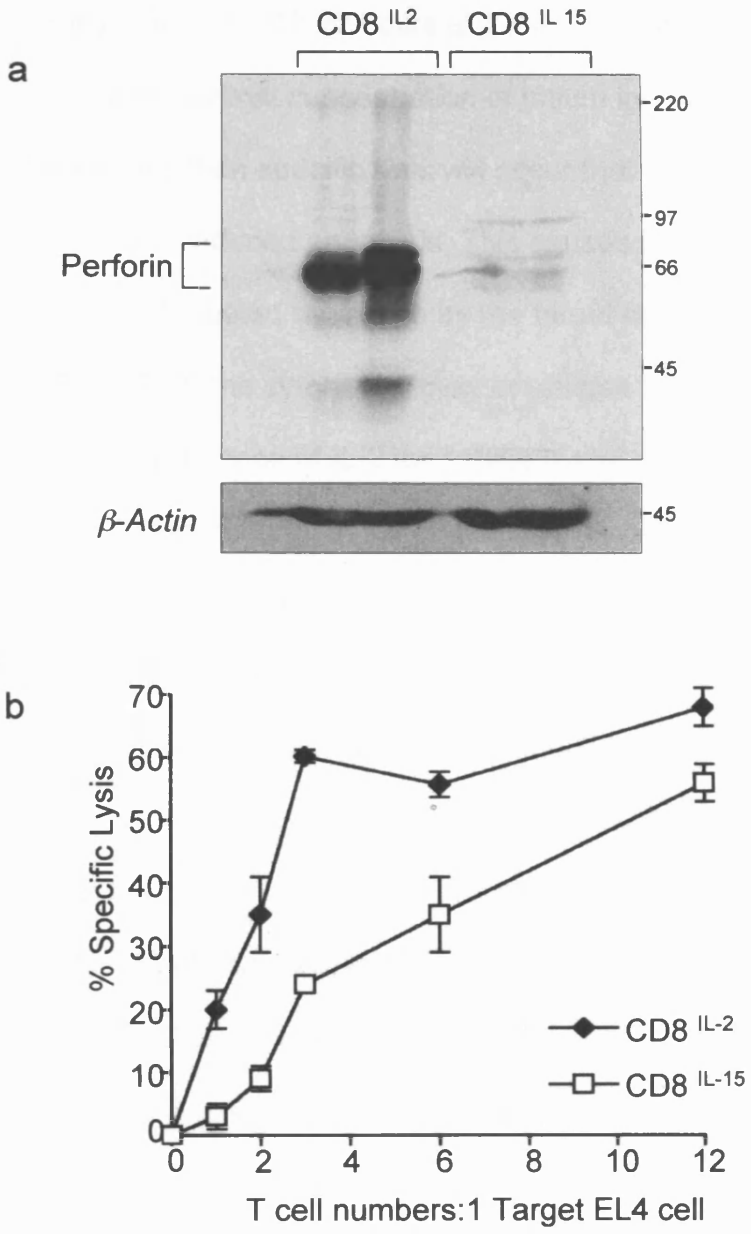


Figure 3.2.4: IL-2 drives differentiation of effector CTL; IL-15 does not. a) Antigen primed P14 CD8+ T cells cultured for 5 days in either 20ng/mL IL-2 ($CD8^{IL-2}$) or IL-15 ($CD8^{IL-15}$) were lysed and immunoblotted for perforin with antibody HuPerf-2d4. β-Acitn was used as a loading control. b) Cytotoxicity assay: target EL4 cells were labelled with ^{3}H-thymidine. T cell lysis of target cells was measured by the loss of tritium.

of H-2D^b class I MHC molecules. The target cells used were cultured at 5 x 10^6 cells/mL in fully complemented RPMI 1640 and incubated with 5μCi/mL of tritiated thymidine (^{3}H-thymidine) for 12-20 hours at 37°C. This labelling process allows the incorporation of a maximal concentration of tritium into cellular DNA. If effector CTL kills these cells then specific lysis will occur that is mediated via a caspase-mediated pathway of induced apoptosis. This causes DNA degradation and the associated release of tritiated thymidine by the target cell. In the experiment shown in **Fig 3.2.4b** the cytolytic activity of antigen activated P14 CD8+ T cells cultured in the presence of IL-2 for 5 days ($CD8^{IL\text{-}2}$) was compared to that of antigen primed T cells cultured in the presence of IL-15 for 5 days ($CD8^{IL\text{-}15}$). The target cells in this assay were loaded with the gp33 LCMV specific peptide. **Fig 3.2.4b** indicates that $CD8^{IL\text{-}2}$ cells were potent cytotoxic killer T cells whereas cells cultured in the presence of IL-15 for 5 days ($CD8^{IL\text{-}15}$) showed reduced capacity to specifically lyse target cells at low T cell: target ratios when compared to the $CD8^{IL\text{-}2}$ cells. Von Andrian and colleagues described the IL-15 cultured cells as having very little cytotoxic potential (48). The present data show that although they lack detectable levels of perforin protein at this time point (**Fig 3.2.4a**) they do have some potential to kill albeit less efficiently that IL-2 cultured cells. This residual killing capacity could be mediated by Fas ligand as flow cytometric analysis revealed that EL4 target cells express Fas antigen (data not shown).

3.3 Discussion

The main aim of the experiments in this chapter was to assess the reliability of system described by Manjunath et al (48) for the generation of large numbers

of central memory-like and effector CD8+ T cells *in vitro* for the biochemical analysis of IL-2 and IL-15 signalling in murine T cells. The data confirm that IL-2 and IL-15 are equivalent mitogens for antigen activated T cells, both able to drive DNA synthesis and clonal expansion at a similar rate. However, IL-2 and IL-15 differ in their control of antigen activated T cell morphology or differentiation: $CD8^{IL-2}$ cells remained large, expressed activation markers and developed efficient CTL activity, whereas $CD8^{IL-15}$ became smaller, expressed memory associated cell surface markers and were unable to develop an equally efficient CTL activity as $CD8^{IL-2}$ cells. Von Andrian and colleges have suggested that antigen activated T cells generated in the *in vitro* cultures with IL-15 are equivalent to memory T cells (48, 51). We did not perform *in vivo* adoptive transfer experiments to compare the immune recall or homing potential of $CD8^{IL-2}$ versus $CD8^{IL-15}$ cells to assess this in more detail. However, *in vitro* transwell assays indicated that $CD8^{IL-15}$ T cells preferentially migrated toward lymphoid-tissue chemokines 6Ckine/CCL21 (SLC) and MIP-3β/CCL19 (ELC) whereas $CD8^{IL-2}$ T cells did not (data not shown). This is consistent with the phenotype described by the studies of the Von Andrian group (51). Whether $CD8^{IL-15}$ cells are a true model for memory T cells is questionable not least because it is based on exposing cells to a single cytokine, which will not reproduce what happens *in vivo*. As well, the concentration of exogenous cytokine added might not represent that encountered *in vivo.* Other factors to consider are the presence of other immune cells *in vivo*, inflammation status of host skewing normal cytokine milieu and migratory status of cells determining what tissue cytokine environment is encountered.

There is also one very important factor to consider about the use of soluble IL-15 to study IL-15 signal transduction. *In vivo* studies of IL-15Rα-/- and IL-15-/- mouse models have indicated that IL-15 is presented in *Trans* by the IL-15R alpha chain to β/γc complexes on T cells thus inducing a signal as an immobilised molecule (207). It is thought that T cells responding to IL-15 do not need to express the IL-15 receptor alpha chain, rather, this chain is required to be expressed by cells producing IL-15 (thought to be dendritic cells, macrophages or bone marrow stromal cells) (523). This is unlike IL-2 which is known to be secreted as a soluble factor and then signal when it binds to IL2Rα/β/γc complexes. *Trans* IL-15 signalling means that cells producing IL-15 in response to inflammatory cytokines such as IFN-γ secrete the soluble form of the cytokine which then associates with the high affinity IL-15Rα chain, signalling is said to occur when the IL-15 presenting cell meets the IL2/15β:IL-2/15γc complex, on an antigen activated T cell (207-209) (210, 212). This theory of *Trans* presentation has been eluded to by Singer and colleges where they propose that homeostatic cytokines such as IL-7 and IL-15, which are of low abundance and expressed in a wide variety of tissues, maybe sequestered on the surface of these tissues to stop cells consuming all available cytokine, as in the case of activated T cell production and consumption of IL-2 for reaction limiting purposes, and allow for maximum exposure for homeostatic survival of immune cells in peripheral and lymphoid tissues (539).

Most studies of IL-15 signal transduction to date have used soluble IL-15 and there has been no comparison of soluble IL-15 versus presented (bound) IL-15 signalling. Presentation of IL-15 in *Trans* to antigen activated T cells could be achieved using an epithelial or bone marrow stromal cells as a feeder cell line,

where cells would be stably transfected with cDNA of IL-15 protein (secreted form); IL-15 + IL-15Rα chain; and IL-15Rα only. These cells could then be co-cultured with P14 LCMV antigen activated CD8+ T cells, which would then be analysed for changes in cell morphology and compared to those seen in the soluble IL-15 system. This would be a very complicated system for biochemical analysis.

Although there might be concerns about the physiological relevance of soluble IL-15 there is a need to be pragmatic. It should also be noted that the framework for antigen receptor signal transduction has been based on the use of soluble TCR antibodies, yet has provided invaluable data that has been subsequently verified in physiological models. Moreover, although there may be some concerns about studying the effects of soluble IL-15 in terms of the physiology of T cell immune responses it should be noted that affects of soluble IL-15 is used clinically for immunotherapy.

Numerous T cell cytokines have now been used in the clinic to expand or activate immune cells or their progenitors, including IL-2 which was approved by the FDA in 1992 and has been used in the clinic regularly in the treatment of cancers, graft rejection/tolerance and immunosuppressive diseases. IL-2 has not really fulfilled its promise as an immunotherapeutic agent mostly due to problems with toxicity and unpleasant side effects. IL-15 appears to be less toxic and is increasingly being considered for clinical use as an alternative to IL-2, because of its shared functions with IL-2, and is now being considered to have a promising future in the treatment of cancer (203). IL-15 remains in pre-clinical trials but seems a likely candidate for exogenous cytokine therapy potentially expanding NK cells, memory CD8+ T cells and some non-classical T

cell lineages. Its value resides in its ability to drive T cell proliferative expansion but not differentiation. Thus IL-15 is being pursued for use in pathogen-targeted vaccination and against cancer antigens (reviewed in (540, 541)) and in the treatment of HIV (542, 543). Hence, comparisons of how soluble/exogenous IL-2 and IL-15 effect T cell physiology and biochemistry will be extremely useful in understanding how these cytokines might be used in the clinic.

Chapter 4

IL-2 and IL-15 differentially regulate T cell growth

4.1 Introduction

One unexplored area of cytokine signal transduction is how cytokines compare in their effects on basic processes of cell growth. The question of how T cell cytokines regulate T cell growth is important because even if different cytokines can induce a common genetic program, differences in how they modify protein synthesis and cellular energy metabolism will influence how this genetic program is executed. The morphology of T cells cultured in IL-2 versus IL-15 shown in the previous chapter indicates that IL-2 and IL-15 may not be equivalent growth factors for T cells. This possibility deserves further investigation because understanding the biochemistry of cell growth control in lymphocytes is essential to understanding the unique actions of different members of the γc cytokine family. It is usually assumed that mitogens are growth factors. The basic fact that cell division or proliferation is intrinsically linked to cell growth seems inevitable, as dividing cells need to generate new membrane. However, it is not clear how cells co-ordinate growth and division to maintain cell size (526, 544-546). There are two basic camps of thought 1. Cell division and growth are intrinsically linked where cell growth is rate limiting for cell division and driven by intersecting and common signalling pathways (547-549). 2. Cell growth can be regulated independently to division by initiating

independent signalling cascades in response to common or independent factors *growth* (526-528). It is clear that cell size at division may be variable and seems to depend on the type of extracellular signals present to whether cell cycle or cell growth or both are maintained, also supported by data in this thesis. Although, a cell needs to reach a minimum size before it can divide. The threshold of size required for division may vary between cell types and rely on quantity and quality of external stimuli. Data from Martin Raff and Sally Leevers support this theory of cell division independent cell growth (526-528). The term cell growth has been used broadly to describe processes of cell division, development and differentiation. However, in this thesis T cell growth is describing the function of T cell volume, mass or size and is aimed at addressing links between this phenomenon and metabolic processes triggered within the cell as a consequence of γc cytokine signal transduction. Accordingly, in the present chapter the actions of IL-2 and IL-15 on protein synthesis and cell growth of antigen activated CD8+ T cells are compared.

4.2 Results

4.2.1 Cytokines IL-2 and IL-15 differentially regulate cell size and protein content.

To measure the change in cell size from naïve T cell to antigen activated T cell we made use of the flow cytometer where light is passed through a single cell suspension and diffracted in response to the size and density, granularity, of a cell passing through the light. This gives a relative measurement of cell size. These measurements are expressed as forward light scatter and side light scatter (FSC, SSC) which can be shown in the form of a two dimensional dot

plot or one dimensional histogram. The data in **Fig 4.2.1a** & **4.2.1b** show both dot plots and histograms of the FSC and SSC of *ex vivo* CD8+ naïve P14 TCR transgenic T cells and antigen activated P14 CD8+ T cells which have been stimulated for two days with the gp33 LCMV specific peptide in the presence of resident splenic antigen presenting cells. Data shows naïve T cells have a low FSC and SSC indicating that they are very small. In contrast, CD8+ T cells activated with specific antigen have both a high FSC and SSC indicating that these cells are dramatically larger and more granular. Flow cytometric analysis of forward and side scatter profiles is a method to estimate cell size and granularity but cannot distinguish whether cells have changed cell volume due to osmotic swelling or whether cells have changed protein content. The size difference between quiescent CD8+ T cells and antigen activated T cells is further defined in **Fig 4.2.1c** where naïve and activated T cells were analysed for cellular protein content. These data clearly show that antigen activation of naïve T cells leads to a dramatic increase in the cellular protein content, approximately 4-fold, and cell size. Images of antigen activated T cells cultured in IL-2 indicate that they are large granular cells whereas cells cultured in IL-15 appear much smaller and more similar in size to naïve T cells.

To further examine the impact of IL-2 and IL-15 on T cell size, ex vivo P14 CD8+ T cells were activated with gp33 peptide for 2 days washed and cultured in the presence of 20ng/mL of IL-2 ($CD8^{IL\text{-}2}$) or IL-15 ($CD8^{IL\text{-}15}$), after 5 days in culture cell size and protein content was assessed. Data in **Fig 4.2.2a** shows cytometric dot plots of FSC/SSC analysis of $CD8^{IL\text{-}2}$ and $CD8^{IL\text{-}15}$ at day 5 in culture. The FSC/SSC profile of $CD8^{IL\text{-}2}$ effector T cells is indicative of large granular cells, comparable to the initial population of antigen activated T lymphoblasts.

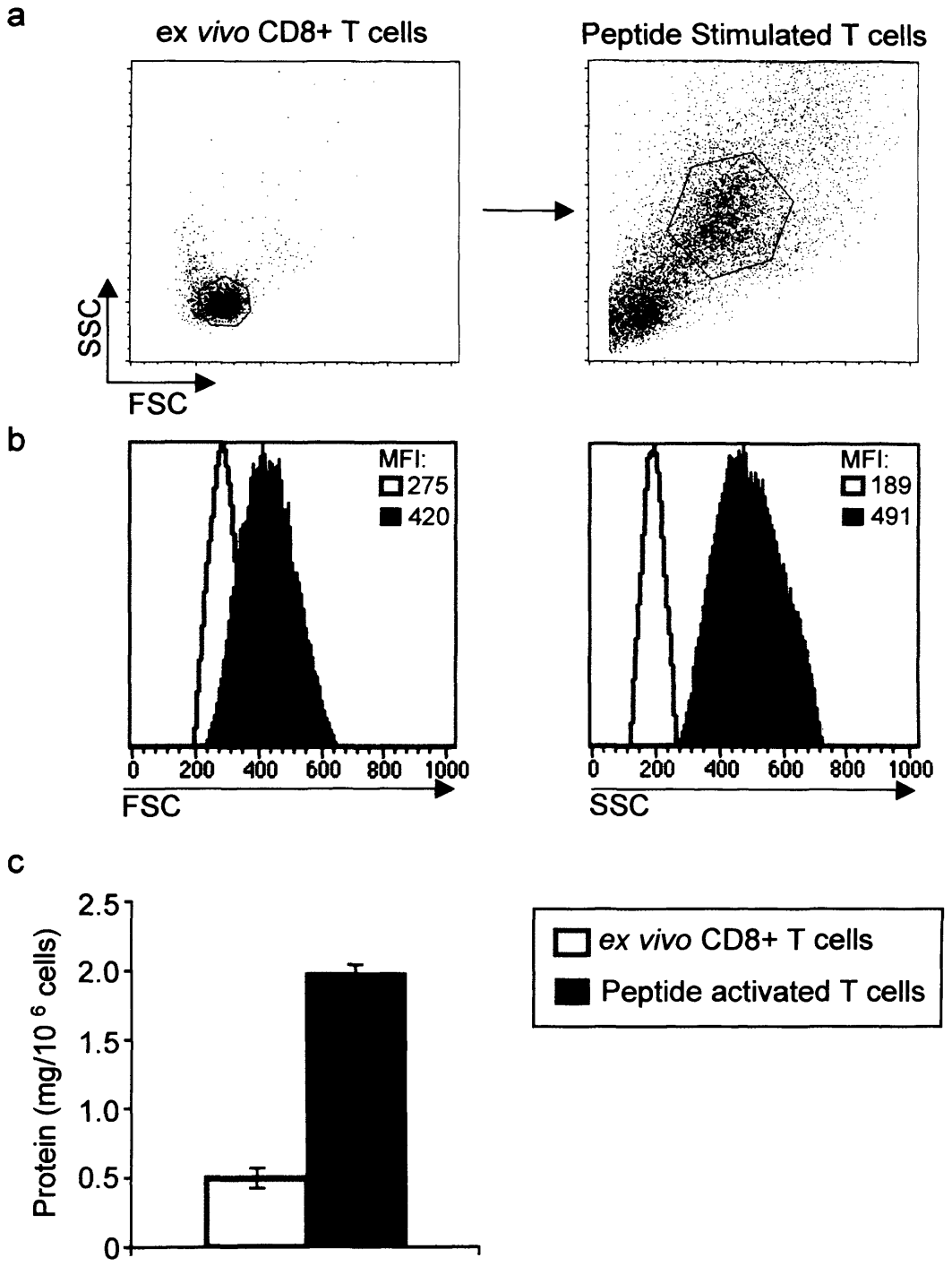


Figure 4.2.1: Antigen stimulation of naïve CD8+ T cells dramatically increases cell size and protein synthesis. a, b) FACS dot plots and histograms (FSC/SSC) of naïve P14 LCMV CD8+ T cells or gp33 antigen activated P14 LCMV CD8+ T cells.c) Cellular protein content (mg) of 10^6 naïve P14 CD8+ T cells or antigen activated P14 CD8+ T cells.

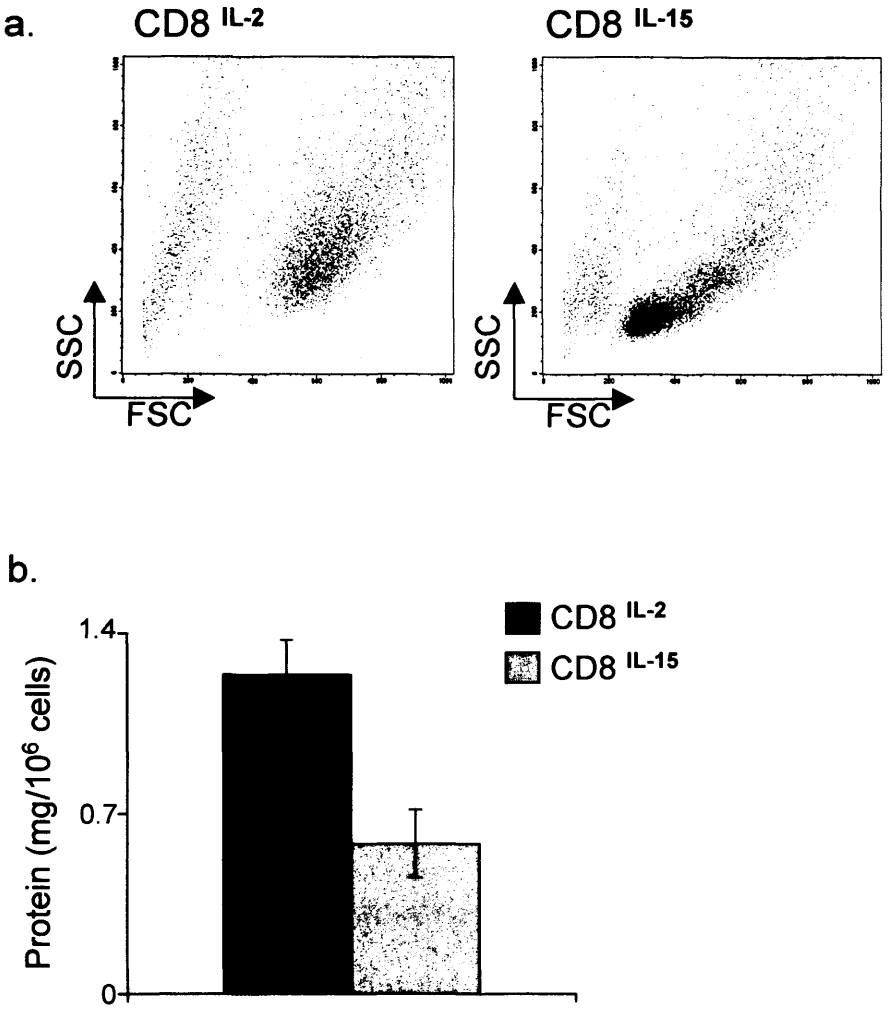


Figure 4.2.2: Cytokines IL-2 and IL-15 differentially regulate cell size and protein content. Naïve CD8+ P14 T cells were activated with gp33 peptide for 2 days, washed and cultured in the presence of 20ng/mL of IL-2 (CD8 $^{IL-2}$) or IL-15 (CD8 $^{IL-15}$). a) Representative FACS dot plots show FSC/SSC and b) protein concentration of CD8 $^{IL-2}$ and CD8 $^{IL-15}$ cells after 5 days in culture.

However, the FSC/SSC profile of activated T cells maintained over the same time period with IL-15 is consistent with these cells being markedly smaller (**Fig 4.2.2a**). The differences in cell size indicated by flow cytometric analysis is also reflected in the protein content of these two populations: antigen primed cells cultured in IL-2 have a higher protein content than cells cultured in IL-15 (**Fig 4.2.2b**). Thus IL-2 is more efficient at maintaining cell size and protein content than IL-15. Data for cells cultured in medium alone was not included as these cells are all dead after 5 days in culture.

Data in **Fig 4.2.3** show the kinetics with which antigen primed T cells cultured in IL-15 reduce in size. The first point to note is that antigen activated T lymphoblasts cultured in medium alone, in the absence of exogenous antigen or cytokine stimuli, rapidly reduce cell size as judged by reductions in their flow cytometric forward and side light scatter (FSC, SSC) profiles (**Fig 4.2.3a**). These changes are seen within 24-48 hours of antigen deprivation. After 48 hours these cells are mostly dead or dying and hence are eliminated from the 72hour time point (**Fig 4.2.3a**). When antigen activated T cells are cultured with IL-2 they maintain cell size (**Fig 4.2.3a**). In contrast, antigen activated T cells cultured in IL-15 decrease in size however, this is not an immediate response as with complete withdrawal of cytokine but occurs over a period of 48-72 hours (**Fig 4.2.3a**). Data in **Fig 4.2.3b** show the protein content of $CD8^{IL-2}$ and $CD8^{IL-15}$ over 24-72hrs and reveal that T cells cultured without exogenous cytokine rapidly reduce their protein content over a 48hour period. It is not possible to look at the 72 hour time point as the cells are all dead by this time (**Fig 4.2.3b**). The first indication that IL-15 cannot maintain the morphology of antigen activated T cells

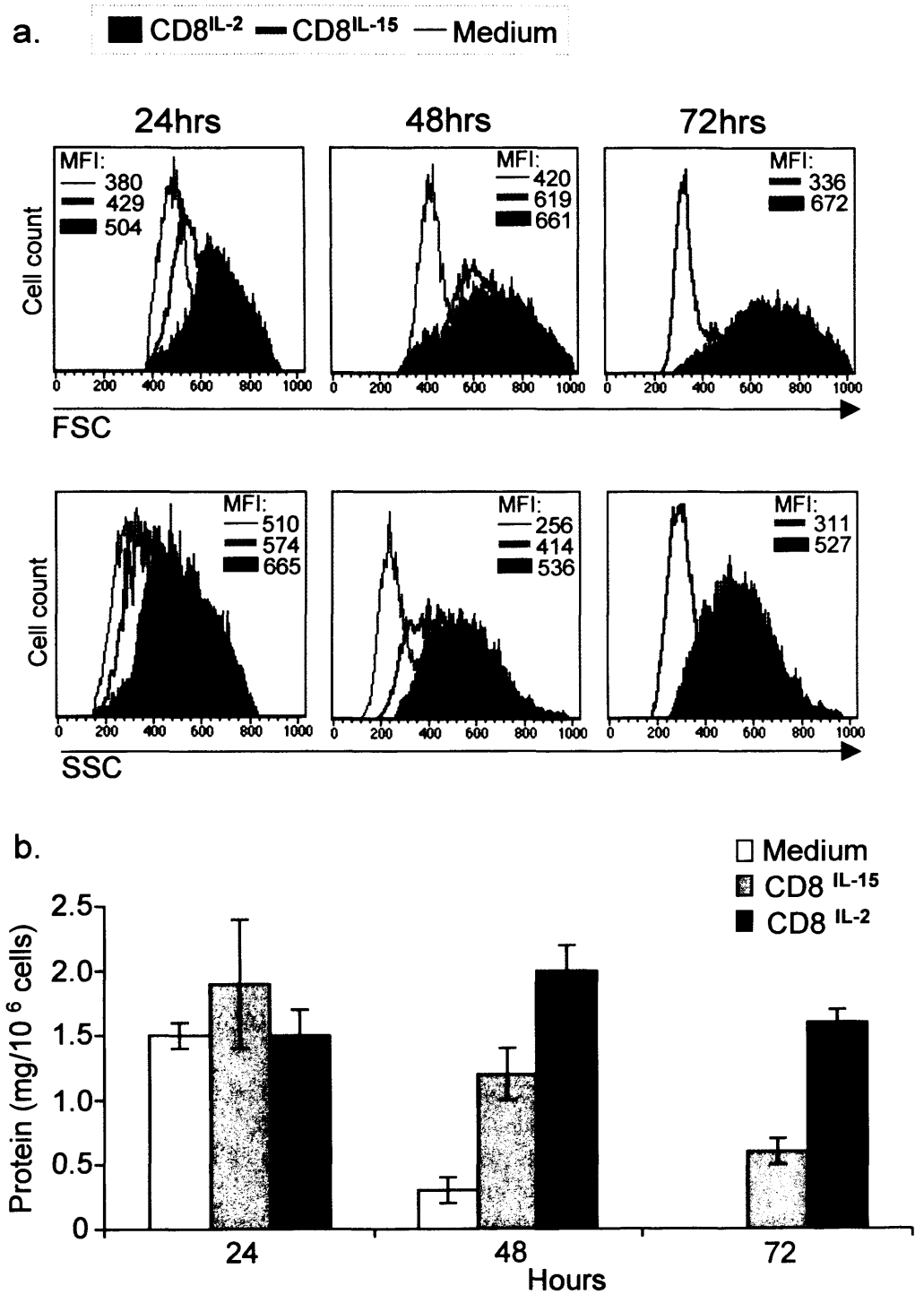


Figure 4.2.3: Kinetics of the change in cell size of antigen activated CD8+ T cells cultured with IL-2 or IL-15. a) FACS histograms of FSC/SSC. b) Protein content of antigen activated P14 LCMV CD8+ T cells cultured for 24, 48 and 72 hours in medium alone, 20ng/mL of IL-2 ($CD8^{IL-2}$) or IL-15 ($CD8^{IL-15}$).

can be judged initially by comparing the side scatter profiles of T cells maintained in IL-2 or IL-15 for 24hours; at this point the cells have similar protein content. Differences in the protein content of antigen pulsed T cells cultured with IL-2 versus IL-15 are slower to emerge but T cells exposed to IL-15 for 48-72 hours have both reduced forward and side light scatter and approximately 1.5-2-fold lower protein content than T cells maintained for an equivalent time in IL-2 (**Fig 4.2.3a & 4.2.3b**).

The changes to the morphology and cell size of antigen activated T cells cultured in IL-15 are not permanent but reversible: IL-15 maintained T cells switched to a culture containing IL-2 rapidly increase in cell size (**Fig 4.2.4**). Suggesting IL-2 as a dominant cytokine in controlling and promoting high rates of T cell growth.

4.2.2 IL-2 and IL-15 differentially regulate protein synthesis.

The ability of IL-2 but not IL-15 to maintain the protein content of antigen activated T cells could be explained if these two cytokines could differentially regulate endogenous amino acid levels and the *de novo* synthesis of protein. Initially, the ability of cytokine treated T cells to incorporate a tritium labelled amino acid mixture was used to quantify rates of amino acid uptake. The data in **Fig 4.2.5a** show that an antigen activated T cell cannot autonomously maintain amino acid uptake when cultured in medium alone, indicated at the zero cytokine point which coincides with a very low level of tritiated (^{3}H) amino acid incorporation. Both IL-2 and IL-15 are able to induce a dose dependent increase in amino acid uptake in antigen activated T cells but strikingly, amino acid uptake in IL-2 activated cells is approximately double that of cells stimulated

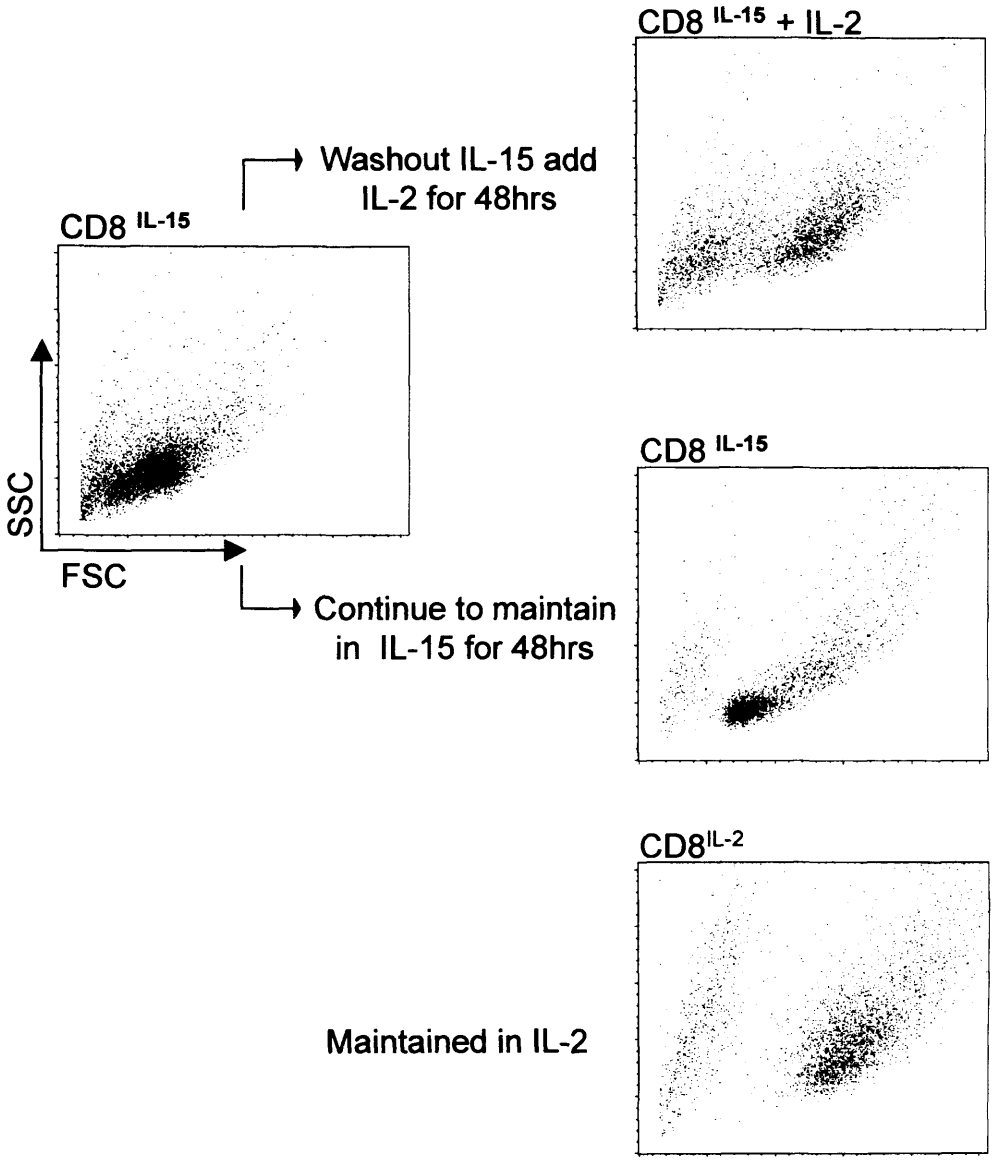


Figure 4.2.4: CD8 $^{IL-15}$ cultured T cells increase size in response to IL-2. FACS dot plots (FSC,SSC) profiles of antigen activated P14 CD8+ T cells cultured in 20ng/mL of IL-15 ($CD8^{IL-15}$) for 3 days. After 3 days $CD8^{IL-15}$ cells were washed and re-cultured in 20ng/mL of IL-2 ($CD8^{IL-15}$ + IL-2) or 20ng/mL IL-15 ($CD8^{IL-15}$) for a further 48 hours. As a comparison, FSC/SSC profiles of activated T cells maintained in IL-2 ($CD8^{IL-2}$) for the entire 5 days is shown.

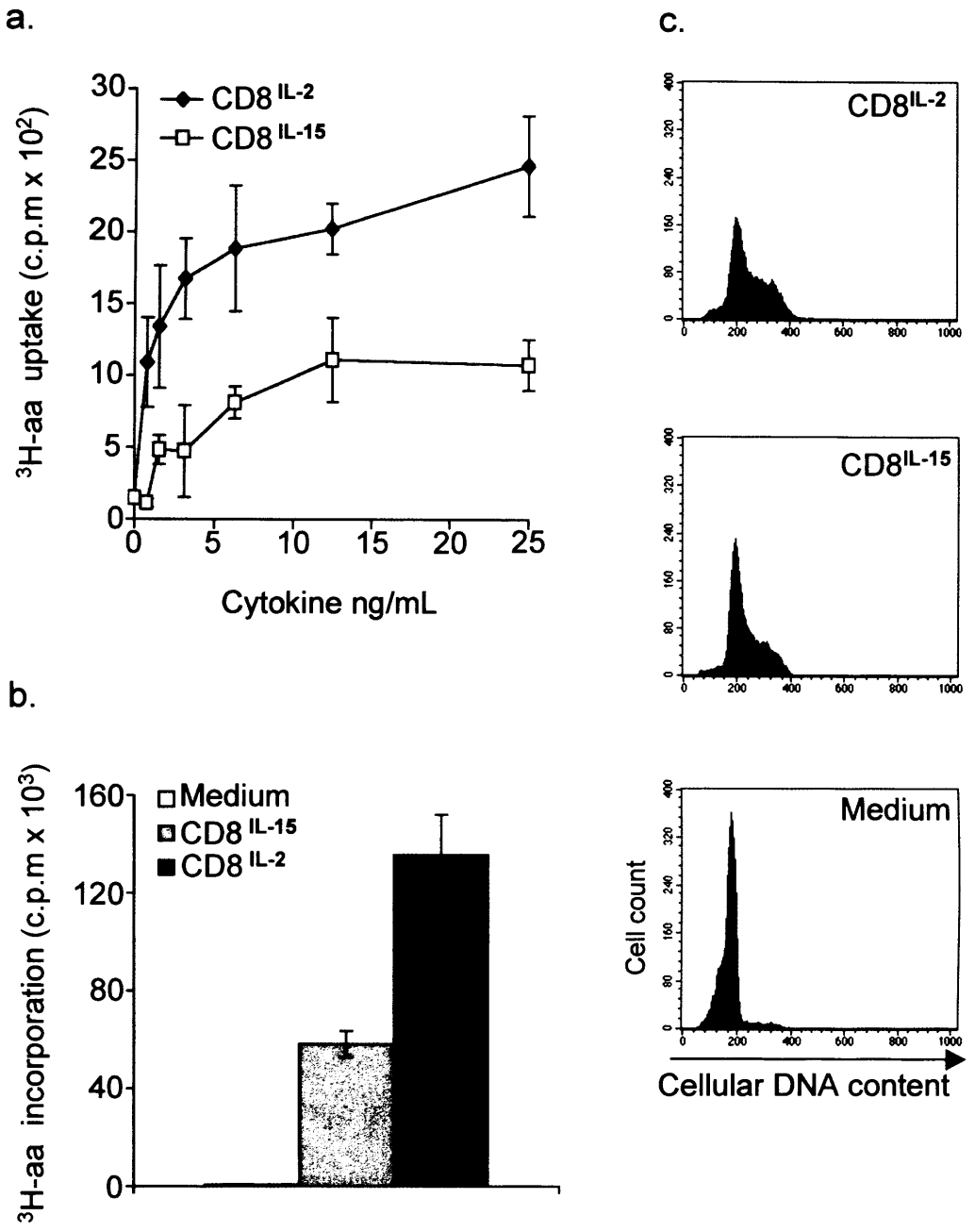


Figure 4.2.5: IL-2 and IL-15 regulate amino acid uptake into cells and incorporation into cellular proteins. a) Tritiated amino acid (^{3}H-aa) uptake by 1 x 10^5 antigen activated P14 CD8+ T cells cultured for 24hours with the indicated concentrations of IL-2 or IL-15. b) Incorporation of tritiated amino acid (^{3}H-aa) into cellular protein of 5 x 10^6 antigen activated P14 CD8+ T cells cultured for 24hours in 20ng/mL IL-2, 20ng/mL IL-15 or medium alone. c) FACS histograms of Hoescht stained cellular DNA content of activated P14 CD8+ T cells cultured for 24hours with medium, 20ng/mL IL-2 or 20ng/mL IL-15.

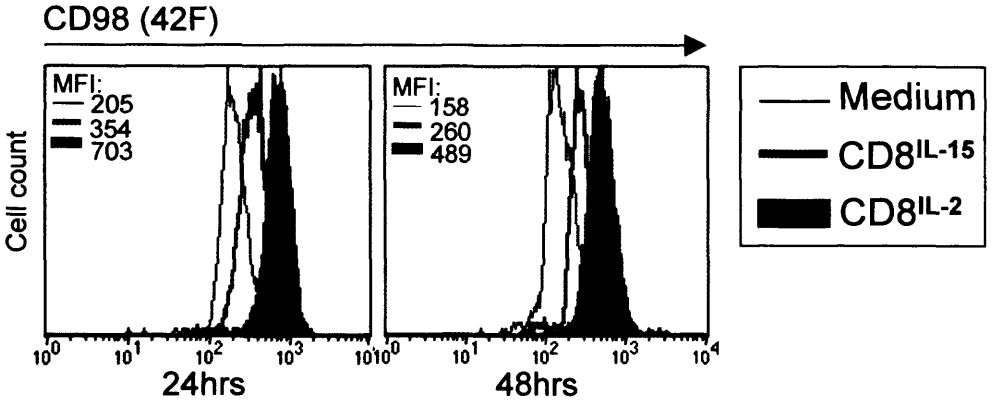
with IL-15 (**Fig 4.2.5a**). The differences between IL-2 and IL-15 in amino acid uptake translated into differences in protein synthesis. Hence, when the ability of these two cytokines to induce incorporation of tritiated (^{3}H) amino acids into cellular proteins was compared, the IL-2 response markedly exceeded the IL-15 response (**Fig 4.2.5b**).

The regulation of cell size is thought to be tightly coordinated to the regulation of cell cycle progression in mammalian cells. However, differences between IL-2 and IL-15 in their ability to regulate cell size of antigen primed T cells is not due to differences in their abilities to initiate DNA synthesis and cell cycle progression. Hence, IL-2 and IL-15 are equivalent mitogens and the frequency of cells in the proliferative (S and G2) phases of the cell cycle is comparable in IL-2 and IL-15 cultured antigen primed T cells (23% and 11% versus 23% and 14% respectively) (**Fig 4.2.5c**).

4.2.3 IL-2 and IL-15 differentially regulate expression of transferrin receptors and amino acid transporters.

Mammalian cells have a broad range of mechanisms for the trans-membrane transport of amino acids. In haematopoietic cell systems, CD98, a marker of activated lymphocytes, is the common heavy chain subunit, which is thought to support the formation of key amino acid transporters in these cells (432). The data in Fig **4.2.6a** show that antigen activated CD8+ T cells maintained in IL-2 have high levels of surface CD98 compared to cells cultured with no cytokine. These data show that antigen activated T cells cannot autonomously maintain surface levels of CD98. IL-15 is able to maintain some expression of CD98 when compared to the control cells cultured in medium alone. However, IL-15 is

a.



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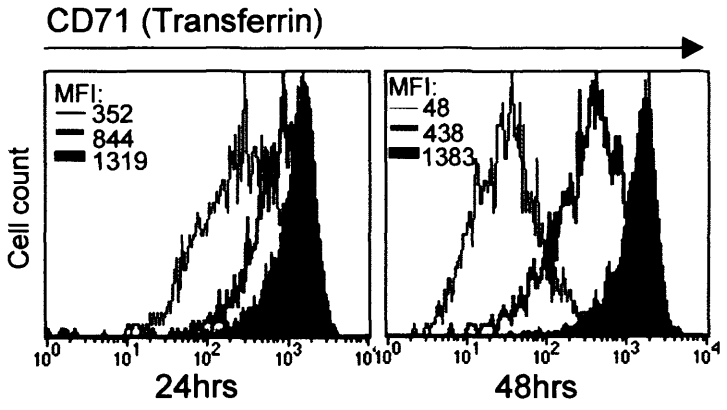


Figure 4.2.6: Expression levels of amino acid transporter CD98 (42F) and transferrin receptor CD71 on T cells cultured in IL-2 or IL-15. FACS histograms of the expression of a) an amino acid transporter subunit CD98 and b) Transferrin receptor CD71 on gp33 antigen activated P14 LCMV CD8+ T cells cultured in 20ng/mL IL-2 ($CD8^{IL-2}$), IL-15 ($CD8^{IL-15}$) or medium alone for 24-48hours.

c.

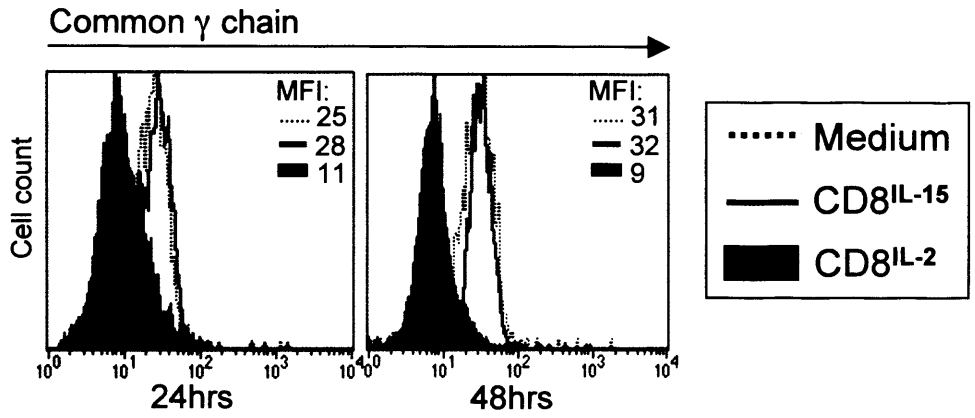


Figure 4.2.6: Expression levels of amino acid transporter CD98 (42F) and Transferrin receptor CD71 on T cells cultured in IL-2 or IL-15. c) FACS histograms show expression levels the common γc chain cytokine receptor subunit on antigen activation P14 CD8+ T cells cultured in 20ng/mL IL-2 ($CD8^{IL-2}$), IL-15 ($CD8^{IL-15}$) or medium alone for 24-48hrs.

not as potent as IL-2 and the data in **Fig 4.2.6a** show that within 24hours of exposure to IL-15, CD98 levels are almost twofold lower than the levels seen in IL-2 maintained cells.

The uptake of amino acids is an energy dependent process and optimal growth responses in cytokine stimulated lymphocytes are associated with up regulation of transferrin receptors which deliver iron, a necessary cofactor for a number of critical metabolic reactions, to the cell interior. Antigen activated T cells maintained in IL-2 express high levels of transferrin receptor (CD71) but these decline rapidly if cells are cultured in medium alone (**Fig 4.2.6b**). The data show that levels of transferrin receptors in T cells maintained in IL-15 are markedly reduced compared to T cells cultured with IL-2 (**Fig 4.2.6b**). The level of transferrin receptor expression in a T cell cultured with IL-15 for 24 or 48hours is higher than in cells cultured with no exogenous cytokine but lower than in T cells cultured in the presence of IL-2 (**Fig 4.2.6b**). T cells cultured in IL-15 do not globally down regulate all surface receptors: levels of the common γc chain cytokine receptor subunit are higher in the presence of IL-15 or medium alone when compared to CD8+ T cells grown in the presence of IL-2 (**Fig 4.2.6c**).

4.3 Discussion

The object of the present study was to compare the role of cytokines as growth factors for T cells. IL-2 and IL-15 are equivalent mitogens for antigen stimulated CD8+ T cells but the present data show they are not equivalent growth factors and are strikingly distinct in their ability to control protein synthesis and T cell size. Antigen activated CD8+ T cells are stimulated by TCR and co stimulatory signals such as CD28 to become large blastoid cells approximately twice as big

as naïve T cells. Herein we show that such antigen induced blasts cannot autonomously maintain their size and are unable to maintain amino acid incorporation or de novo protein synthesis without exogenous cytokine stimulation. Both IL-2 and IL-15 are able to induce protein synthesis in antigen activated T cells, but protein synthesis rates in IL-2 activated cells are approximately two fold of those in IL-15 stimulated cells. The differential action of IL-2 and IL-15 on amino acid uptake and protein synthesis is associated with differences in the ability of these two cytokines to maintain surface levels of the transferrin receptor CD71 or the amino acid transporter subunit CD98. The difference in the ability of IL-2 and IL-15 to induce protein synthesis is approximately 2 fold, which may not seem like a huge difference, but IL-2 and IL-15 are potent and equivalent mitogens and a two fold difference in protein synthesis in rapidly dividing cells will quickly translate into a significant difference in the size of daughter cells and subsequent progeny.

Thus IL-2 is a strong stimulus for T cell growth whereas IL-15 is much weaker. The ability of IL-2 to induce cell growth was thought to be associated with its role as a mitogen. However the level of cell growth induced by IL-2 exceeds that required for mitosis, as judged by the ability of IL-15 to sustain mitosis and T cell clonal expansion without maintaining T cells at the same size as IL-2. Hence the potency of IL-2 as a growth factor may be more relevant to its ability to sustain synthesis and production of effector molecules in cytotoxic T cells. The differential effect of IL-2 and IL-15 on general protein synthesis may resolve the puzzle of how IL-2 and IL-15 differentially direct CD8+ T cell fate (51, 229), even though they initially activate common signal transduction pathways and induce very similar patterns of gene transcription (205, 206). The differentiation of

effector CTL takes several days and to sustain the production of cytokines or cytotoxic mediators, effector lymphocytes will need to maintain high rates of cell metabolism for a prolonged period. A sustained two fold difference in protein synthesis over a period of many hours or days would considerably diminish the ability of activated T cells to produce and secrete effector cytokines and chemokines with profound consequences for T cell differentiation. The power of IL-2 in terms of it's ability to induce protein synthesis could thus explain why it is required to sustain T cell expansion *in vivo* even though it is not essential to initiate T cell cycle progression (129, 529). Moreover, it also gives some insight as to why this cytokine may have a unique role *in vivo* as a regulator of peripheral immune homeostasis (132). In particular, the potency of IL-2 in terms of its ability to drive protein synthesis could explain why antigen stimulated T cells fail to secrete effector cytokines in the absence of IL-2 function even though they appear to undergo normal cell divisions (133).

In the immune system the adaptive CD8+ T cell response specifically seeks and destroys virally infected cells, this response if not controlled can continue and may eventually cause unwanted collateral damage, which can lead to the development of autoimmunity. IL-2 is associated with increased susceptibility of effector T cells to programmed cell death or apoptosis and this may be part of the key immunoregulatory role of IL-2 *in vivo*. The present results showing the potent growth stimulating ability of IL-2 compared to IL-15 may explain why this cytokine is associated with increased susceptibility of effector T cells to apoptosis. T cells exposed to IL-2 would thus expend high amounts of energy to maintain their effector status and this may lead to a state of metabolic exhaustion that promotes susceptibility to cell death. When comparing the

effects of IL-15 on CD8+ T cells it was surprising to find that even though this cytokine is an equivalent mitogen for T cells it is not as potent a growth factor as IL-2 in the fact that it cannot support massive cell size and protein synthesis. However, cells grown in IL-15 are able to divide at a similar rate to cells grown in IL-2 whilst maintaining a lower rate of protein synthesis. Proliferative expansion with IL-15 would thus be much less energy demanding than proliferative expansion with IL-2 and could explain why IL-15 does not promote T cell apoptosis in the same way that IL-2 does.

The object of the present study was to compare the role of cytokines as growth factors for T cells. IL-2 and IL-15 are equivalent mitogens for antigen stimulated CD8+ T cells but the present data show they are not equivalent growth factors and are strikingly distinct in their ability to control protein synthesis and T cell size. Antigen activated CD8+ T cells are stimulated by TCR and co stimulatory signals such as CD28 to become large blastoid cells approximately twice as big as naïve T cells. The data show that such antigen induced blasts cannot autonomously maintain their size and are unable to maintain amino acid incorporation or *de novo* protein synthesis without exogenous cytokine stimulation (shown in medium only data). Both IL-2 and IL-15 are able to induce protein synthesis in antigen activated T cells, however, protein synthesis rates in IL-2 stimulated cells are approximately two fold of those in IL-15 stimulated cells. Hence even though the difference is small between the abilities of IL-2 and IL-15 to sustain protein synthesis, when taken into consideration with the fact that both IL-2 and IL-15 are equivalent mitogens, a two fold difference in protein synthesis in rapidly dividing cells will quickly translate into a significant difference in the size of subsequent daughter cells.

The initial differences in cell size, protein synthesis and amino acid uptake between antigen activated cells cultured in IL-2 and IL-15 within over 24-72 hours could be associated with differences in the ability of these two cytokines to maintain surface levels of the transferrin receptor CD71 and the amino acid transporter subunit CD98. Transferrin receptors transport iron into the cell, which is an essential molecule in mediating cellular reactions.

Thus IL-2 seems to be a potent stimulus for T cell growth whereas IL-15 is much weaker in the context of regulating the availability of amino acids and iron to the cell. The ability of IL-2 to induce cell growth was thought to be associated with its role as a mitogen. However the level of cell growth induced by IL-2 seems to be in excess of that required for mitosis, as judged by the ability of IL-15 to sustain mitosis and T cell clonal expansion without maintaining T cells at the same size as IL-2. Hence the potency of IL-2 as a growth factor may be more relevant to its ability to sustain synthesis and production of effector molecules in cytotoxic T cells. The differential effect of IL-2 and IL-15 on general protein synthesis may resolve the puzzle of how IL-2 and IL-15 differentially direct CD8+ T cell fate (51, 229), even though they initially activate common signal transduction pathways and induce very similar patterns of gene transcription (205, 206).

The differentiation of effector CTL takes several days and to sustain the production of cytokines or cytotoxic mediators, effector lymphocytes will need to maintain high rates of cell metabolism for a prolonged period. A sustained two fold difference in protein synthesis over a period of many hours or days would considerably diminish the ability of activated T cells to produce and secrete effector cytokines and chemokines with profound consequences for T cell

differentiation. Hence, the essential need for IL-2 signalling in T cells may relate to it's potent ability to induce protein synthesis, which is intrinsically required to sustain T cell expansion *in vivo* even though it is not essential to initiate T cell cycle progression (129, 529). Moreover, it also gives some insight as to why this cytokine may have a unique role *in vivo* as a regulator of peripheral immune homeostasis (132). In particular, the potent ability of IL-2 to drive protein synthesis could explain why antigen stimulated T cells fail to secrete effector cytokines in the absence of IL-2 function even though they appear to undergo normal cell divisions (132).

In summary, the present study demonstrates that cytokines that are equivalent mitogens can have different potency in terms of regulating T cell growth or protein synthesis. IL-2 and IL-15 have very different actions as inducers of protein synthesis and T cell growth although they are equivalent in their ability to drive T cell cycle progression.

The maximal level of protein synthesis induced by IL-2 is in excess of what is needed for T cell mitosis and may explain the unique non-redundant role for this cytokine in the mammalian immune response.

Chapter 5

A mechanism of regulating T cell growth: *PI3K inhibitor LY294002 effects cell size and protein synthesis in antigen activated T cells cultured with IL-2 and IL-15 by regulating nutrient uptake.*

5.1 Introduction

T cell growth is intrinsically linked to the ability of a cell to produce or synthesise protein, and it's capacity to access sources of energy essential to cellular process from extracellular stores.

Previous data in this thesis suggests that antigen activated T cells can modulate their cell size or volume in response to growth factors IL-2 and IL-15 and that these processes are independently regulated from the cells ability to divide or proliferate. This distinction between T cell 'growth' and 'proliferation' challenges the current understanding of cell size regulation and presents the possibility that this process can be regulated independent from cell cycle and division in activated T cells. Thus, the aim of the present chapter was to explore signalling pathways involved in growth regulation mediated by IL-2 and IL-15.

One evolutionarily controlled pathway involved in cell growth regulation is mediated by the lipid product of class I phosphoinositide 3-kinases (PI3Ks),

Phosphatidylinositol (3,4,5) triphosphate ($PI(3,4,5)P_3$). For example, the activation of naïve murine CD8+ T cells by antigen/MHC complexes on APC causes a sustained accumulation of $PI(3,4,5)P_3$ and an increase in T cell size (32, 550). An inhibitor LY294002, which binds the ATP binding pocket of PI3K to block its catalytic activity prevents this accumulation of $PI(3,4,5)P_3$ and prevents antigen induced blastogenesis (32).

Similarly, the ability of CD28 to induce glucose metabolism and an increase in growth of human T cells is blocked by PI3K inhibitors (437). There is also genetic evidence that PI3K effector pathways are important for the regulation of T cell mass: the deletion of PDK1 in T cell progenitors in the thymus causes a reduction in size (410). As well, the expression of a constitutively active mutant of PKB in T cell transgenic mice causes a modest increase in cell size of naïve T cells (436). In this respect, it has been shown in pre B cells that a PI3K/PKB pathway regulates expression of CD98, an important subunit of amino acid transporters (432). In the previous chapter, we have shown that IL-2 and IL-15 differ in their ability to regulate surface expression of CD98 in T cells. If PI3K regulation of CD98 expression also occurs in T cells then it would be predicted that PI3K would be an important regulator of cell growth in IL-2 or IL-15 stimulated T cells.

5.2 Results

5.2.1 Antigen activated T cells cultured with cytokines IL-2 and IL-15 can proliferate and are viable in the presence of PI3K inhibitor LY294002 for an extended period of time.

To dissect the role of PI3K in the regulation of cellular growth responses in antigen activated T cells responding to IL-2 and IL-15, experiments were conducted using the PI3K inhibitor LY294002. IL-2 and IL-15 are potent survival factors for antigen primed CD8+ T cells. PI3K has been shown to be important for IL-2 regulation of T cell cycle progression (505) and in many cell lineages, PI3K signals are known to be crucial in mediating cell survival responses. Thus, prior to looking at the effect of PI3K inhibitors on T cell size it was important to establish the effects of the PI3K inhibitor LY294002 on T cell proliferation, survival and viability.

Naïve P14 LCMV splenic T cells were activated in the presence of 1μM gp33 specific peptide for two days, washed free of exogenous antigen and subsequently cultured in medium only, 20ng/mL IL-2 or IL-15 plus or minus 10μM LY294002 for extended periods of time.

To assess effects of LY294002 on T cell proliferation, cell counts were taken at 72hours of culture, using trypan blue viable dye (**Fig 5.2.1a, 1c**). These results show that LY294002 inhibits but does not abrogate IL-2 or IL-15 induced proliferation. T cells cultured in the presence of IL-2 and IL-15 proliferated at an approximate 2 fold higher rate than cells in the presence of LY294002, however, even though cells cultured in the presence LY294002 did have a reduced cell numbers the remaining cells were proliferating at a rate approximately five times

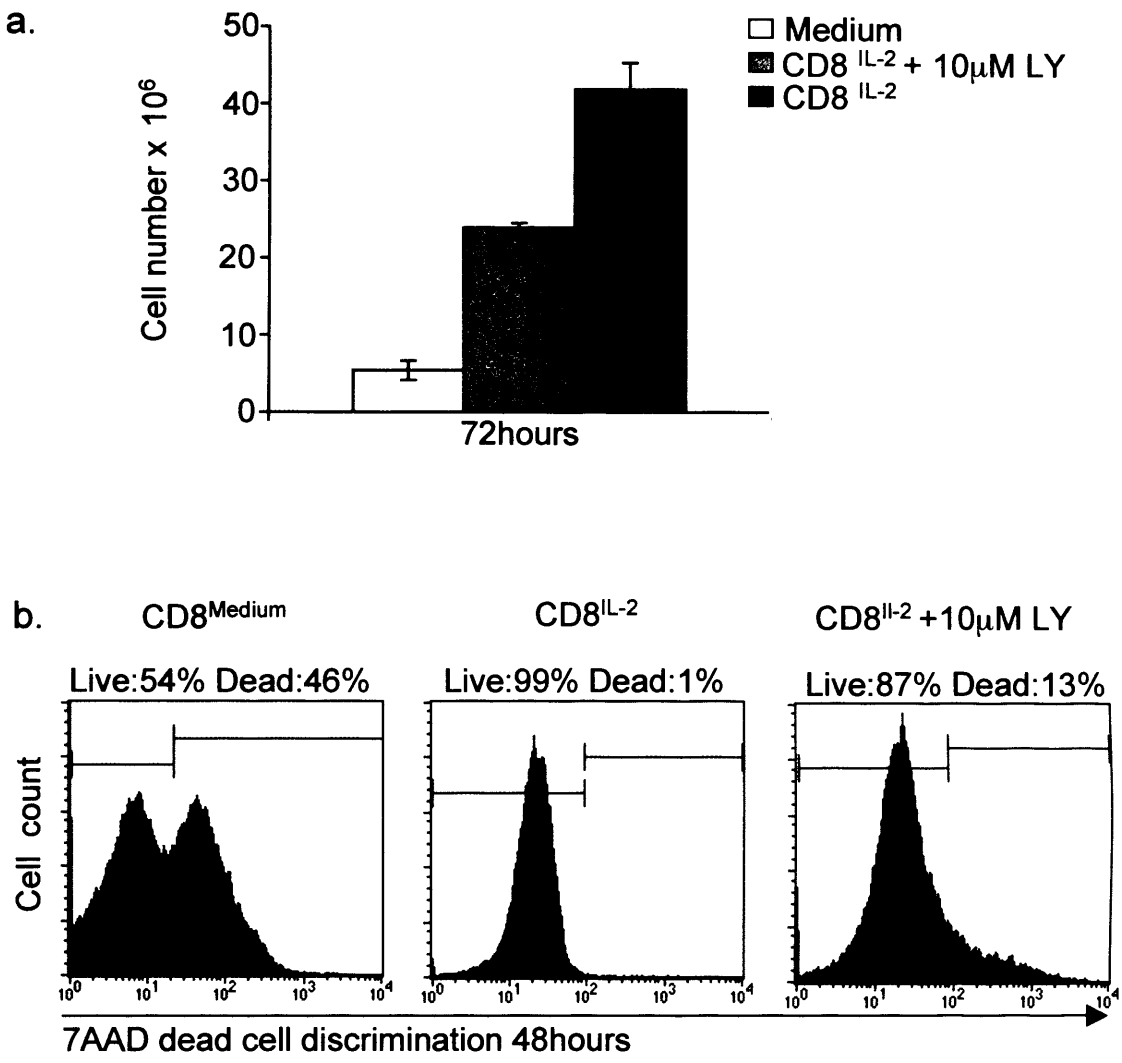
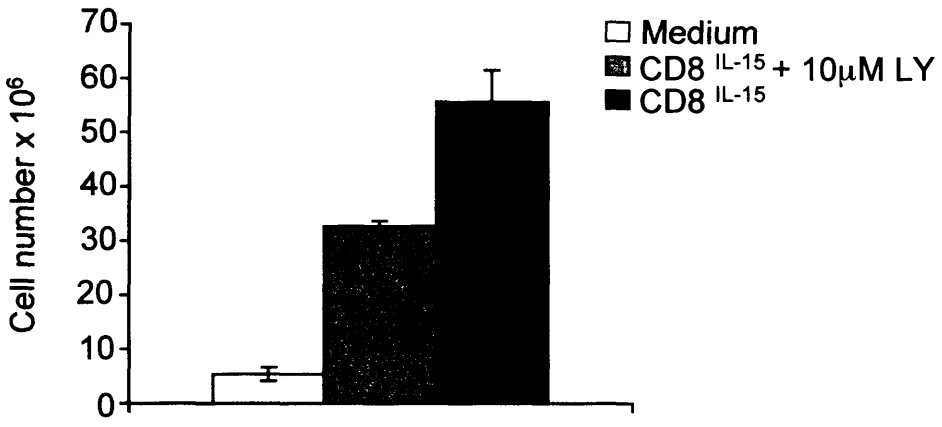
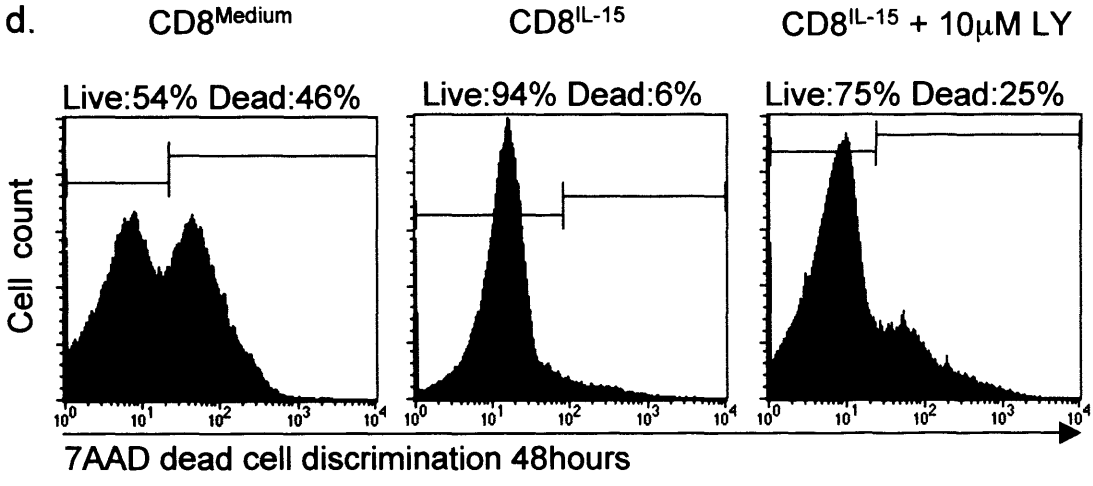


Figure 5.2.1: Antigen activated T cells cultured in the presence of 20ng/mL IL-2 or IL-15 plus and minus 10μM LY294002 are viable and proliferate. a, c) Cell count of antigen activated P14 CD8+ T cells cultured for 72hrs in medium only, 20ng/mL IL-2/IL-15 or IL-2/IL-15 + 10μM LY294002. b, d) Histograms indicating percentages of live and dead cells determined by 7AAD staining at 48hours of these cultures.

c.


Cell number x 10^6
70
60
50
40
30
20
10
0
Medium
CD8 IL-15 + 10μM LY
CD8 IL-15

d.


CD8Medium
CD8IL-15
CD8IL-15 + 10μM LY
Live:54% Dead:46%
Live:94% Dead:6%
Live:75% Dead:25%
Cell count
7AAD dead cell discrimination 48hours

that of cells cultured without stimuli, or medium alone (**Fig 5.2.1a, 1c**). In further experiments flow cytometry was used to analyse cell death or viability of T cells cultured in the presence of the PI3K inhibitor. In these experiments the dye 7AAD was used to quantify cell death, this dye is excluded from viable cells but absorbed by dead or dying cells. **Fig 5.2.1b** shows activated T cells cultured in the presence of 20ng/mL IL-2, 20ng/mL IL-2 plus 10μM LY294002 or in medium alone for 48hours. Cells cultured in IL-2 are 99% viable as compared to only 40-50% viability of cells cultured without cytokine. T cells cultured in the presence of IL-2 plus LY294002 were 87% viable. LY294002 thus has an impact on T cell viability but this is minor compared to the effects of removing IL-2 (**Fig 5.2.1b**). The data in **Fig 5.2.1d** show cell viability of activated T cells cultured in 20ng/mL IL-15, 20ng/mL IL-15 plus 10μM LY294002 or in medium alone. $CD8^{IL-15}$ cells were 94% viable whereas $CD8^{IL-15}$ + LY294002 cultured cells were 75% viable compared to 54% viability in cells cultured in medium alone (**Fig 5.2.1d**). The inhibition of PI3K thus seems to have an impact on the viability of T cells cultured in IL-15 than IL-2 however, both IL-2 and IL-15 are able to induce substantial cell survival and some proliferation in the absence of PI3K activity.

5.2.2 PI3K inhibitor LY294002 mediates a reduction of cell size in antigen activated T cells cultured in either IL-2 or IL-15.

Flow cytometry was used to assess the effects of PI3K inhibitor LY294002 on activated T cell size. Forward and side scatter (FSC/SSC) dot plots and histograms indicate the relative size of gp33 peptide activated CD8+ P14 LCMV T cells cultured in IL-2 (**Fig 5.2.2**) or IL-15 (**Fig 5.2.3**) in the presence or absence of LY294002. The data in **Fig 5.2.2** shows that the ability of IL-2 to

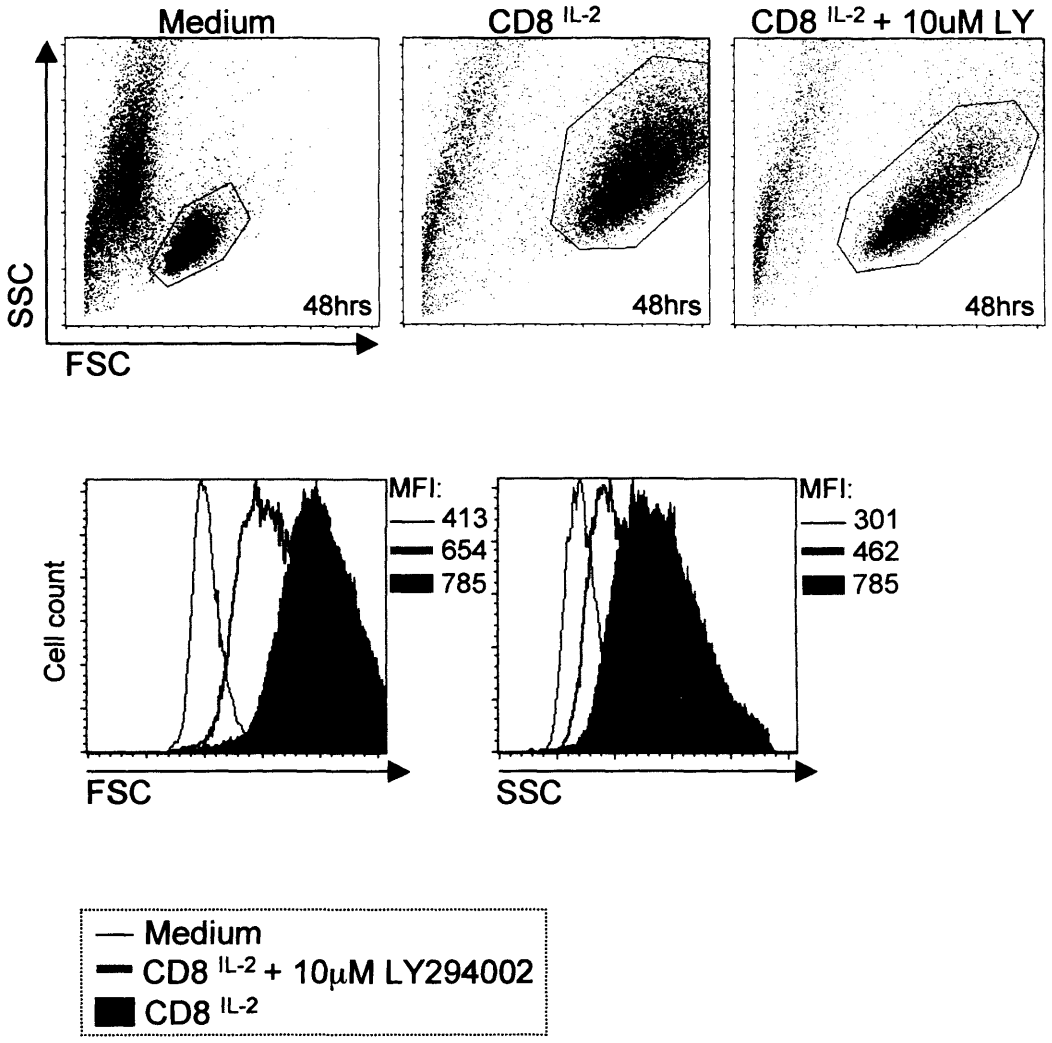


Figure 5.2.2: Antigen activated T cells cultured in the presence of IL-2 + 10μM LY294002 are smaller than T cells cultured in IL-2 alone. FACS dot plots and histograms show FSC/SSC profiles of antigen primed P14 CD8+ T cells maintained for 48 hours in medium, 20ng/mL IL-2 ($CD8^{IL-2}$) or 20ng/mL IL-2 ($CD8^{IL-2}$) plus 10μM LY294002 a PI3K inhibitor.

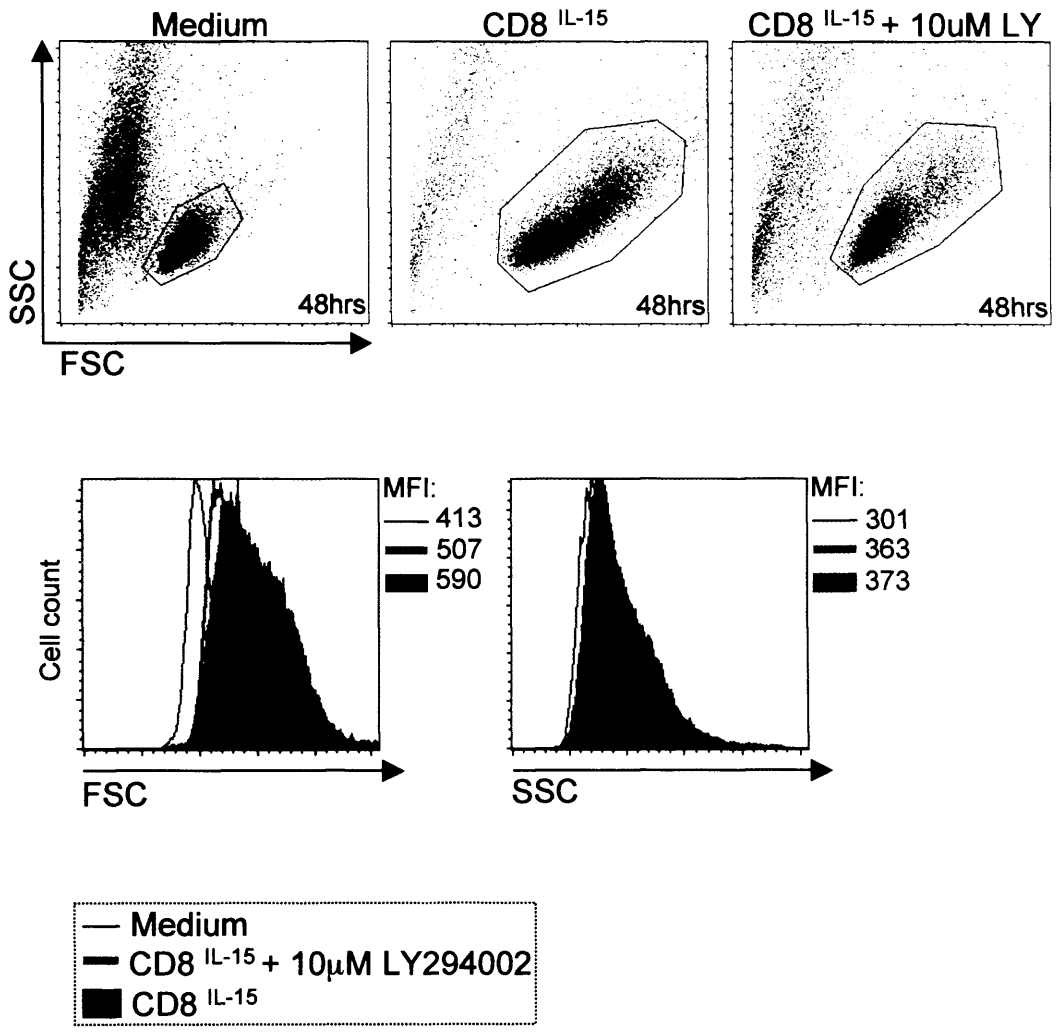


Figure 5.2.3: Antigen activated T cells cultured in the presence of IL-15 + 10μM LY294002 are slightly smaller than T cells cultured in IL-15 alone. FACS dot plots and histograms show FSC/SSC profiles of antigen primed P14 CD8+ T cells maintained for 48 hours in medium, 20ng/mL IL-15 ($CD8^{IL-15}$) or 20ng/mL IL-15 ($CD8^{IL-15}$) plus 10μM LY294002 a PI3K inhibitor.

regulate T cell size is LY294002 sensitive: IL-2 activated T cells treated with LY294002 are smaller than cells exposed to IL-2 alone. **Fig 5.2.3** shows the same effect on cells cultured in IL-15 with or without LY294002 present, again suggesting $CD8^{IL-15}$ cultured cells are also LY294002 sensitive in the context of cell size. These data suggest that PI3K may play a dominant role in the regulation of T cell size.

5.2.3 IL-2 and IL-15 regulation of protein synthesis and T cell growth is disrupted in the presence of LY294002.

T cell growth or T cell size is directly linked to the rate at which a cell can produce protein. To assess the role of PI3K in the ability of IL-2 and IL-15 to regulate protein synthesis, the impact of the PI3K inhibitor LY294002 on the ability of IL-2 and IL-15 to regulate protein synthesis was examined.

Data in **Fig 5.2.4** show that LY294002 inhibited IL-2 induced amino acid incorporation into cellular protein and amino acid uptake into cells. Congruently, measuring cellular protein content also indicated that treatment with LY294002 reduced the cells ability to synthesize protein by 1.5-2 fold (**Fig 5.2.5**).

Data in **Fig 5.2.6** and **Fig 5.2.7** show the effects of LY294002 on IL-15 induced amino acid uptake and protein content in activated T cells. Like IL-2, IL-15 driven protein synthesis is restricted by inhibiting PI3K activity with LY294002 (**Fig 5.2.6, 7**). Even though it is obvious that PI3K plays a role in mediating protein synthesis down stream of IL-2 and IL-15 in antigen activated T cells, it should also be noted that these cytokines still have a limited capacity to induce protein synthesis and cell growth in the presence of PI3K inhibitors. Where, amino acid uptake, incorporation into cellular protein and overall protein content

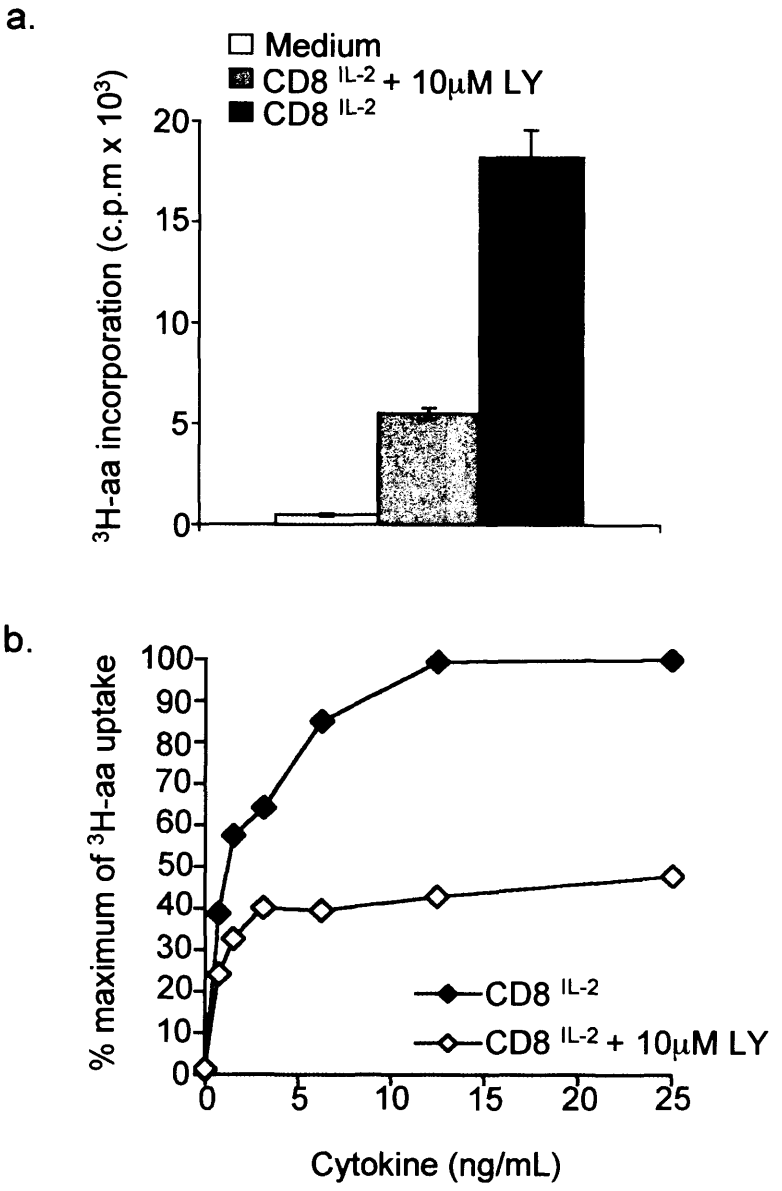


Figure 5.2.4: Culturing antigen activated T cells in the presence of IL-2 + 10μM LY294002 impedes the cells ability to synthesize protein when compared to cells cultured in IL-2 alone. a) Incorporation of tritiated amino acids (^{3}H-aa) into precipitated cellular protein of antigen primed P14 CD8+T cells maintained for 48 hours in medium, 20ng/mL IL-2 or 20ng/mL IL-2 plus 10μM LY294002 a PI3K inhibitor (c.p.m x 10^6 cells). b) Percent maximum values of tritiated amino acid (^{3}H-aa) uptake by antigen primed P14 LCMV CD8+ T cells maintained for 48 hours in medium or the indicated concentration of IL-2 plus or minus 10μM LY294002.

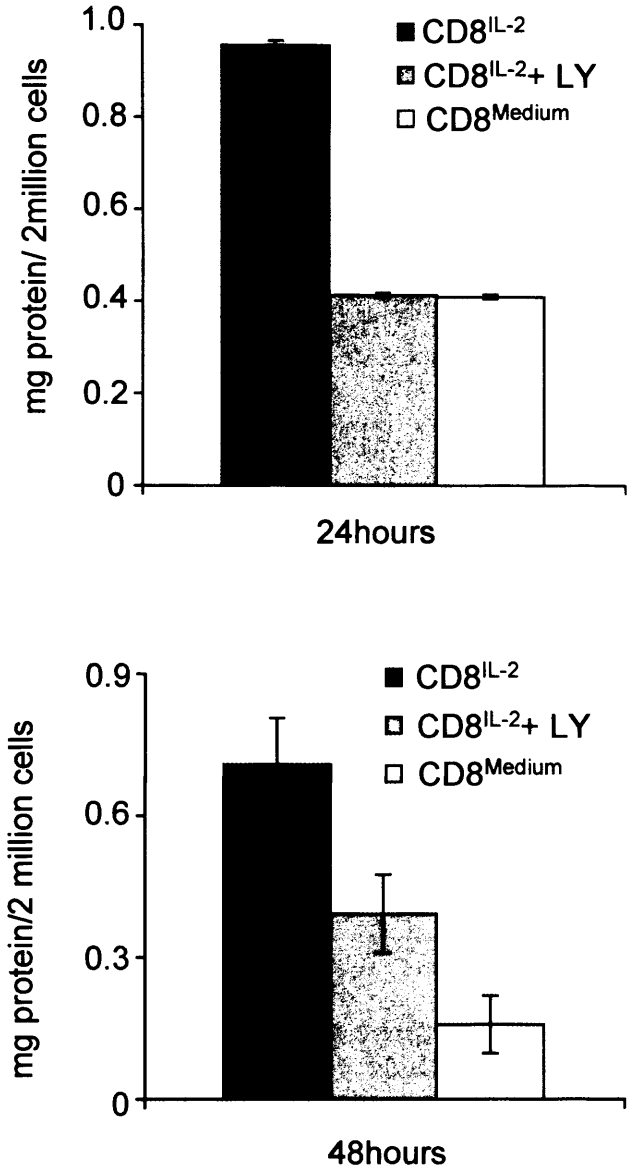


Figure 5.2.5: Antigen activated CD8+ T cells cultured with IL-2 in the presence of 10μM LY294002 contain less protein. Antigen activated P14 CD8+ T cells cultured with 20ng/mL IL-2, 20ng/mL IL-2 + 10μM LY294002, or medium alone were lysed and protein concentrations read in triplicate after 24 and 48hours in culture.

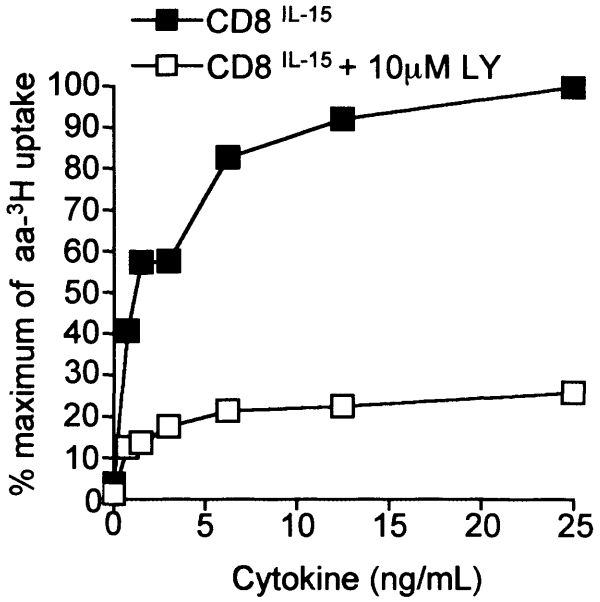


Figure 5.2.6: Culturing antigen activated T cells in the presence of IL-15 + 10μM LY294002 effects protein synthesis when compared to cells cultured in IL-15 alone. Percent maximum values of tritiated amino acid (^{3}H-aa) uptake by antigen primed P14 LCMV CD8+ T cells maintained for 48hours in medium or the indicated concentration of IL-15 plus or minus 10μM LY294002 a PI3K inhibitor

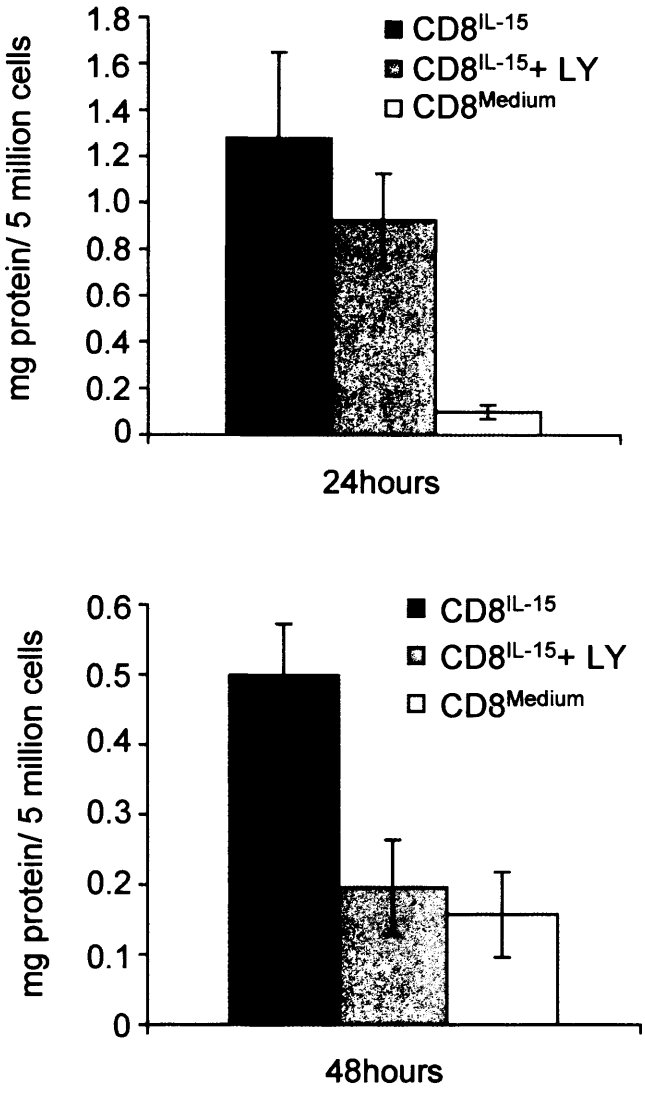


Figure 5.2.7: Antigen activated CD8+ T cells cultured with IL-15 in the presence of PI3K inhibitor 10μM LY294002 contain less protein. Antigen activated T cells cultured with 20ng/mL IL-15, 20ng/mL IL-15 + 10μM LY294002, or cultured in medium alone were lysed and protein concentrations read in triplicate after 24 and 48hours in culture.

levels are reduced but not to that of medium only control cells (**Fig 5.2.4, 5, 6, 7**).

5.2.4 IL-2 and IL-15 induced regulation of transferrin receptor and amino acid transporter expression is LY294002 sensitive.

The PI3K effector PKB/Akt regulates surface expression of CD98, a component of a key amino acid transporter complex in haematopoietic cell (432). We therefore addressed the role of PI3K signalling in expression patterns of CD98 in the context of LY294002 inhibitory effects on antigen activated cells responding to IL-2 and IL-15 over 24 and 48hours.

The data in **Fig 5.2.8a** show that antigen activated CD8+ T cells maintained in IL-2 have high levels of surface CD98 compared to cells cultured with no cytokine. However, these levels are reduced when cells are cultured in the presence of LY294002. Antigen activated CD8+ T cells cultured with IL-15 are also shown to have high levels of CD98 when compared with medium controls, and again decreased CD98 levels in the presence of LY294002 (**Fig 5.2.9a**).

The decrease in CD98 levels on treatment with LY294002 in $CD8^{IL\text{-}15}$ T cells is not as pronounced when compared to IL-2 cultured cells, most likely due to the reduced overall concentration of CD98 receptors on the surface of $CD8^{IL\text{-}15}$ cells when compared with $CD8^{IL\text{-}2}$ (**Fig 5.2.9a**).

The uptake of amino acids is an energy dependent process and optimal growth responses in cytokine stimulated lymphocytes are associated with up regulation of transferrin receptors which deliver transferrin loaded with iron, a necessary cofactor for a number of crucial metabolic reactions, to the cell interior. Antigen

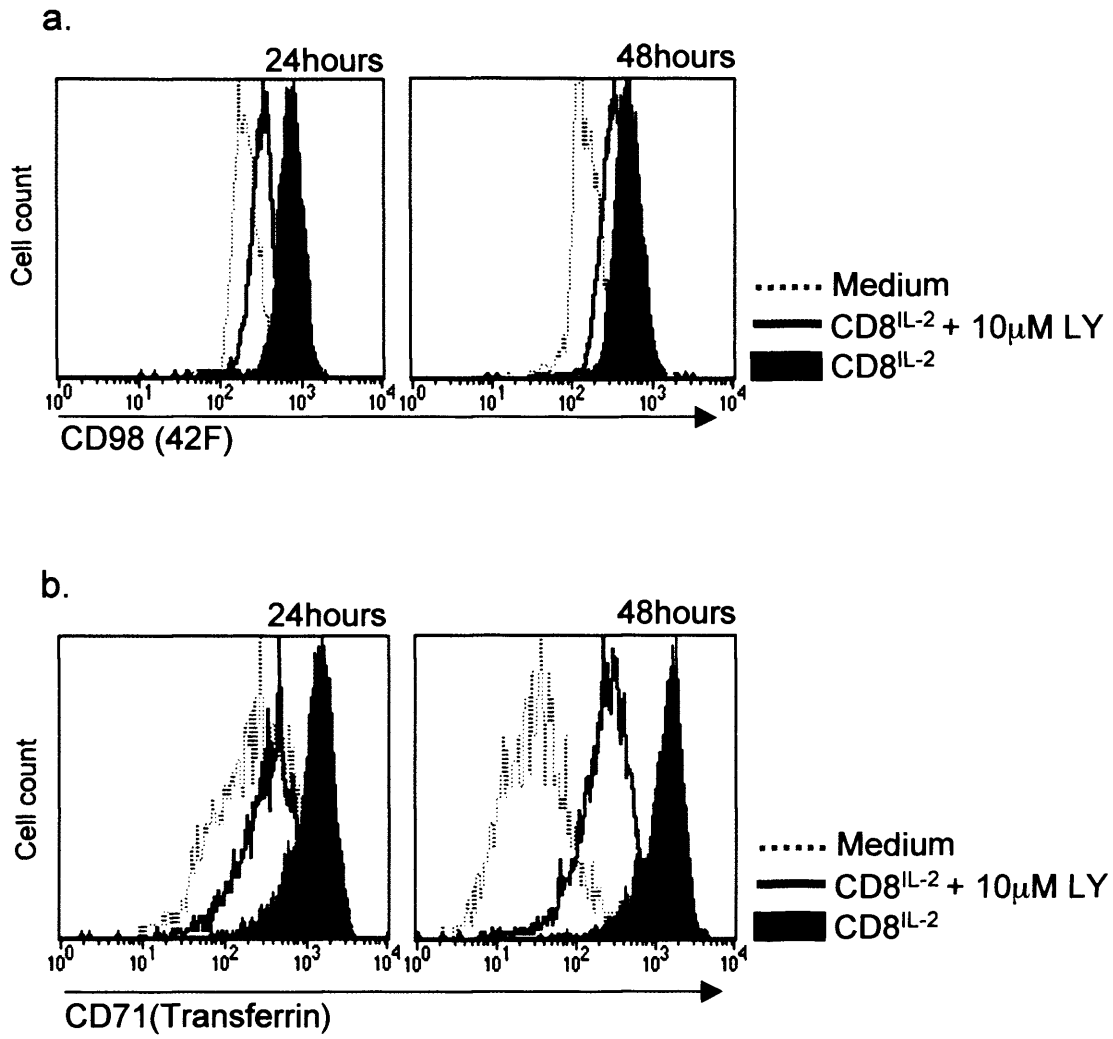


Figure 5.2.8: IL-2 regulation of amino acid transporter subunit CD98 (4F2) and transferrin receptor CD71 is LY294002 sensitive. FACS histograms of a) the amino acid transporter subunit (CD98) and b) transferrin receptor (CD71) expression on the surface of antigen primed P14 CD8+ T cells cultured in medium alone or 20ng/mL IL-2 ($CD8^{IL-2}$) plus or minus 10μM LY294002 a PI3K inhibitor for 24-48hours.

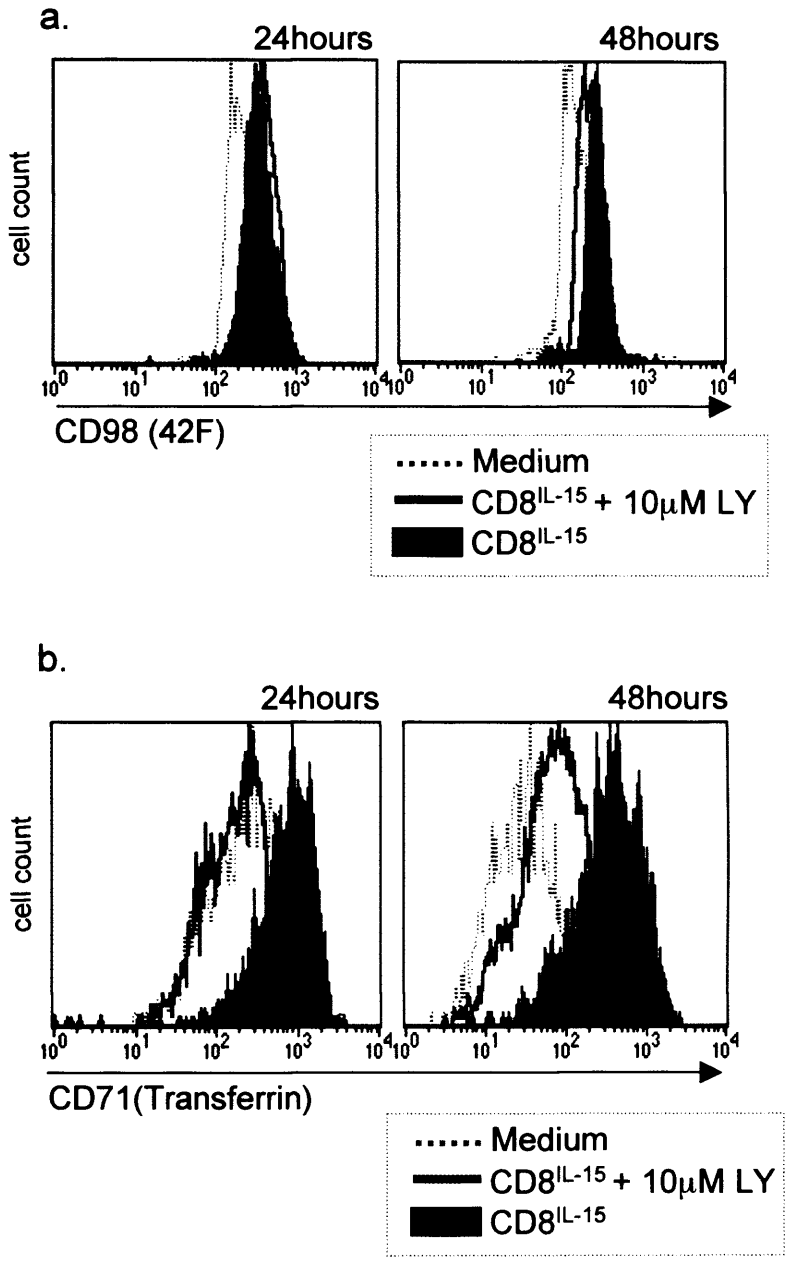


Figure 5.2.9: IL-15 regulation of amino acid transporter subunit CD98 (4F2) and the transferrin receptor CD71 is sensitive to LY294002. FACS histograms of a) amino acid transporter subunit (CD98) and b) transferrin receptor (CD71) expression on the surface of antigen primed P14 CD8+ T cells cultured in medium alone or 20ng/mL IL-15 ($CD8^{IL-15}$) plus or minus 10μM LY294002 after 24 and 48hours in culture.

activated T cells maintained in IL-2 express high levels of transferrin receptor (CD71) over 24-48hours in culture, but these levels decline rapidly if cells are cultured in medium alone (**Fig 5.2.8b**). Transferrin receptor expression is also reduced on activated T cells cultured with IL-2 and PI3K inhibitor LY2494002 (**Fig 5.2.8b**). Antigen activated T cells cultured in IL-15 express high/intermediate levels of CD71 over the 24/48hour time period, respectively, and these levels decrease rapidly when these cells are cultured in medium alone (**Fig 5.2.9b**). Levels of CD71 in IL-15 cultured cells are also reduced rapidly in the presence of LY294002 (**Fig 5.2.9b**).

Hence, data suggests that cytokine induced protein synthesis and amino acid uptake in activated T cells is sensitive to treatment with LY294002, a PI3K inhibitor, suggesting these processes are partly dependent on PI3K activity. Also, cytokine regulation of an amino acid transporter CD98 and transferrin receptor CD71 is LY294002 sensitive, suggesting that IL-2 and IL-15 regulate T cell growth processes via a PI3K driven mechanism controlling the uptake of nutrients.

5.2.5 IL-2 regulation of T cell size is sensitive to wortmannin.

PI3K activity can also be inhibited by a compound wortmannin. This compound physically binds PI3K differently than binding to LY294002 and is known to have a very short half-life in culture, approximately 20 minutes, making it difficult to use in a long-term culture system. Experiments were initially undertaken using LY294002 as it was thought to be the best and most suitable inhibitor at that point. However, after this data was collected crystallographic evidence that

LY294002 sterically binds and inhibits the PIM kinase family of proteins in a similar way to the PI3K inhibition was published (551).

Because recent data from Craig Thompson's lab and others show the involvement of PIM kinases in the regulation of cell size (508, 512, 513) we had to consider the possibility that the effect of LY294002 on T cell size was mediated via its effects on PIM kinases. We therefore did some additional experiments using a second PI3K inhibitor wortmannin to measure the involvement of PI3K in this *in vitro* system. Initial experiments deducing effects on size were completed, however, not all experiments were repeated due to time restrictions.

Thus, relative cell size of antigen activated CD8+ T cells cultured in medium alone, 20ng/mL IL-2, 20ng/mL IL-2 plus 10μM LY294002 or 100nM wortmannin for 48hours was measured by flow cytometry. FACS dot plots and histograms show FSC and SSC, where cells cultured in the presence of LY294002 and wortmannin both have reduced FSC and SSC (**Fig 5.2.10**). These data show that LY294002 and wortmannin have similar effects on T cell size suggesting PI3K plays a role in regulating T cell size.

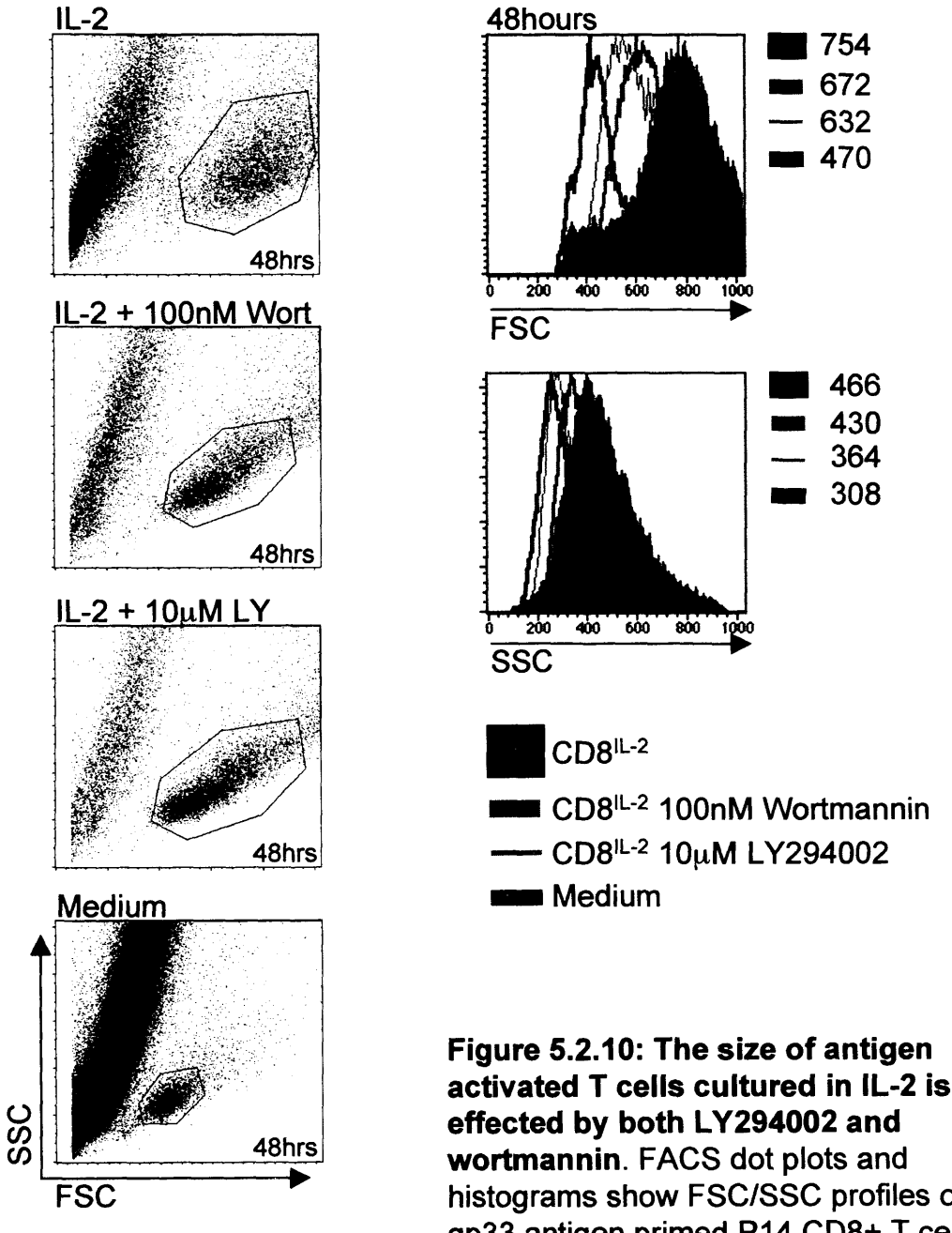


Figure 5.2.10: The size of antigen activated T cells cultured in IL-2 is effected by both LY294002 and wortmannin. FACS dot plots and histograms show FSC/SSC profiles of gp33 antigen primed P14 CD8+ T cells maintained for 48 hours in medium, 20ng/mL IL-2 ($CD8^{IL-2}$) or 20ng/mL IL-2 ($CD8^{IL-2}$) plus 100nM wortmannin, or 10μM LY294002 (PI3K inhibitors)

5.3 Discussion

The present data show that the PI3K inhibitor LY294002 inhibited the ability of IL-2 and IL-15 to regulate T cell size. The effect of LY294002 on size was most obvious in the context of IL-2 responses. Data also show that LY294002 inhibits the capacity of IL-2 and IL-15 to drive amino acid uptake and protein synthesis in CD8+ T cells. Protein synthesis and amino acid uptake is dependent on the availability of amino acids transported into cells by specific amino acid transporters. The results in chapter 4 showed that IL-2 and IL-15 regulate cell surface expression of CD98 a common subunit of amino acid transporters known to mediate transport of neutral and dibasic amino acids across the membrane (552-555), (556). Protein synthesis is also very energetically demanding and accordingly IL-2 and IL-15 up regulate surface expression of CD71 the transferrin receptor, important for iron transport. Data in this chapter shows that IL-2 and IL-15 regulation of CD71 and CD98 is LY294002 sensitive, suggesting that PI3K plays a role in nutrient availability for antigen experienced CD8+ T cells.

The requirement for PI3K activity for protein synthesis in T cells correlates with a PI3K requirement for amino acid uptake. It is however very likely that PI3K has other relevant targets that are important for protein synthesis. Firstly, protein synthesis is energetically demanding and it has been described that PI3K and its effector PKB regulate glucose uptake and hence control ATP generation. Reports have indicated that PI3K/Akt regulates Glut1 glucose transporter in lymphoblastic and naïve T cells in response to γc cytokines and TCR

stimulation. For example Barata et al showed IL-7 mediated Glut1 up-regulation, cell size and iron transport increases as a direct result of PI3K activity in lymphoblastic T cells (363). Craig Thompson's group has also shown Glut1 up-regulation as a consequence of CD28 co-stimulation in naïve T cells and clearly showed the requirement of PI3K in this process via the use of inhibitors (437). Evidence out side the immune system in the context of insulin signalling also support a role for PI3K in controlling glucose transporters Glut1 and Glut4 (557). Thus it seems clear that PI3K/PKB plays a role in modulating T cell glucose metabolism in response to both TCR/CD28 stimulation and γc cytokines.

Little is known of the processes directly regulating glucose transporter expression in T cells although it might be possible to draw conclusions from what is known about insulin regulation of glucose transport. It is thus thought that Glut1 and Glut4 expression is modulated by insulin via the rapamycin sensitive pathway PI3K/PKB/mTOR/4EBP1. Insulin has been shown to up-regulate Glut1 translation via activation the mammalian target of rapamycin (mTOR) and inactivation of its primary substrate 4EBP1, a translation repressor (557).

PI3K activation is required and sufficient for activation of the serine kinase PKB (391). In the IL-3 dependent cell line FL5.12 expression of a constitutively active mutant of PKB can substitute for IL-3 induced expression of CD98 and is also a potent stimulus for cell growth (432).

What is the evidence that PKB is the key mediator for IL-2 and IL-15 induced cell growth? Firstly, it has been noted that pre-T cells lacking the expression of PDK1, a key upstream kinase for PKB, are smaller than normal (410). Secondly,

transgenic mice expressing an active mutant of PKB in naïve T are larger than normal indicating a role for PKB in the control of T cell size (436). However, because the loss of PDK1 blocks T cell development in the thymus we know nothing about the role of PDK1 in IL-2 and IL-15 responses in the periphery (410). Moreover, PDK1 is required for activation of a number of serine kinases not just PKB (396) so it cannot be excluded that any role for PDK1 in the regulation of T cell size is mediated by PDK1 substrates such as the PKCs or RSK and is nothing to do with PKB. Naïve T cells that express an active PKB mutant may be slightly larger that normal but they are nowhere near the size of an IL-2 or IL-15 lymphoblast which indicates that PKB is probably not sufficient for IL-2 or IL-15 regulation of T cell growth.

One evolutionarily conserved nutrient-sensing molecule involved in PI3K control of cell growth in a number of cellular systems is mTOR (mammalian target of rapamycin (reviewed in (455, 558)). mTOR is a serine threonine kinase specifically inhibited by the immunosuppressant rapamycin (456, 559) and is known to play a central role in integrating signals from nutrients (amino acids and energy) and growth factors (in higher eukaryotes) to regulate cell growth and cell cycle progression. TOR senses cellular amino acid (560) and energy or ATP levels (471) and is considered a 'gatekeeper' coupling nutrient availability with cell growth (559) (456).

The nutrient sensing mechanism of TOR remains poorly understood but appears to involve a protein complex containing Raptor (496), mediating rapamycin sensitive TOR functions including phosphorylation of S6K and 4E-BP1. Rictor is a new binding partner for TOR thought to mediate rapamycin insensitive functions (561) although upstream kinases and substrates of TOR-

rictor are still being discovered. Although recent data suggests that the TOR-rictor complex phosphorylates PKB at ser473 facilitating subsequent Thr308 phosphorylation by PDK1 and thus full activation status (400, 503). It has also been implicated in regulation of cellular cytoskeleton (503, 562).

The role of TOR in cell growth has recently been shown in Drosophila where the inactivation of TOR or its substrate S6 kinase results in reduced cell size and embryonic lethality, illustrating the critical role for TOR in cell growth control (563). The functions of mammalian TOR (mTOR) have also recently been dealt with in mice by the disruption of the kinase domain in mTOR. Heterozygote mice were normal and fertile, homozygote embryos died shortly after implantation due to impaired cell proliferation (563). Furthermore, embryonic stem cells deleted for mTOR kinase activity were reduced in size and proliferation arrested (563). These data suggest a central role for TOR in cell growth in mammalian cells. However, we have compared the effect of LY294002 and rapamycin on IL-2 and IL-15 regulation of T cell growth and found very little effect of rapamycin on T cell size compared to the effects of LY294002 although rapamycin did effect T cell proliferation (data not shown). Hence the rapamycin sensitive TOR pathway is necessary for IL-2 induced T cell proliferation but not for T cell growth. It has recently been shown that mTOR is also part of a distinct complex defined by the novel protein rictor (rapamycin-insensitive companion of TOR) (503). The role of the TOR/rictor complex as a regulator of T cell growth is not known. Nevertheless, the failure of rapamycin to block IL-2 and IL-15 induction of protein synthesis argues that the ribosomal S6 kinases (S6K) are not absolutely necessary for cytokine regulation of protein synthesis. Hence PI3K activation is

required and sufficient for S6K1 activation (389) yet rapamycin treatment has very little effect on protein synthesis and cell size of IL-2 or IL-15 stimulated effector CD8+ T cells (data not shown).

Interestingly, the early studies of rapamycin action that showed this drug was good at blocking the induction of T cell proliferation in primary T cells but could not down regulate an ongoing proliferative response (506). One explanation for the selective role of rapamycin in T cell proliferation is that it blocks the initiation of ribosomal biogenesis, induced by activation of naïve T cells, but it does not have a major impact on sustaining ribosome function once the first phase of ribosome biogenesis is complete (564). Ribosome biogenesis must be essential during the initial activation of T cells by antigen/APC because it is hard to imagine that the huge increase in protein synthesis that occurs following immune stimulation of naïve T cells could be achieved without massive ribosomal biogenesis. In this respect there is evidence that the co-stimulatory molecule CD28 regulates T cell activation via rapamycin sensitive mechanisms (565). This is however quite a controversial point with other experiments arguing that rapamycin does not always block CD28 responses (566)

One interesting set of molecules to explore in the context of IL-2 and IL-15 regulation of cell growth are the Pim kinases which have recently been identified by Craig Thompson's lab as important regulators of T cell metabolism (513). Pim kinases are serine/threonine kinases expressed in haematopoietic cells. Their expression is rapidly induced in T cells following stimulation through the TCR or with cytokines like IL-2 and most likely IL-15 although this has not been formally

shown as yet. There are three members of the Pim family Pim1, 2, and 3. Pims were first discovered as a frequent site for proviral insertion in the lymphomas that arise following infection with Murine Maloney Leukemia virus (567, 568). These proteins have a well-documented role in oncogenesis (reviewed in (569, 570)) as deregulation of these genes has been found in many lymphoid tumours. Pims are thought to be transcriptionally and post-transcriptionally regulated in response to T cell activation (571). IL-2 has been shown to induce Pim1 mRNA expression, detected in microarray analysis of immediate early genes (572).

The essential role of Pim kinases in T cells has only recently been recognised. It is now known that constitutive expression of Pim1 reconstitutes thymic cellularity in γc -/- and Rag-/- mice (511) but gene deletion of the single isoforms of PIM kinases has no discernable impact on T cell responses. However, the recent production of mice deficient in Pim1, 2 and Pim3 has yielded important new insights about the relevance of the Pim kinases in T cells. Pim1, 2, 3 triple knockout mice are viable and fertile but with a much reduced body size compared to normal (508). T cell differentiation and export from the thymus was normal in Pim deficient mice although peripheral T cells showed a reduced proliferative response to suboptimal anti-CD3 activation in the presence of IL-2 due to a reduced capacity to undergo cell division. No enhanced apoptosis was observed. Pim-deficient T cells also expressed lower levels of IL-2Rγc chain but did not show reduction in STAT5 signalling. The B cell compartment of these mice showed a more severe phenotype with reduced numbers in the spleen,

increased pre-pro-B cell progenitors in the marrow and a decreased proliferaitve capacity in the presence of IL-7 (508).

T cells deficient in Pim1and Pim2 have been studied by Craig Thompson's lab. These studies have revealed a very interesting ability of Pim kinases to substitute for rapamycin/TOR signals in the regulation of T cell survival, size, blastogenesis (predominantly Pim2 mediated) and proliferation *in vitro and vivo* (513). The key fact to emerge from the work by Thompson is that in normal T cells the regulation of cell survival and growth or size is rapamycin independent whereas in Pim2 deficient T cells these response become rapamycin sensitive (513).

In view of these recent results it would be very interesting to explore the role of PI3K in the regulation of the expression of Pim1 and Pim2. Is it possible that PI3K pathways regulate Pim kinase expression. Little is known about the transcriptional mechanisms that induce expression of Pim kinases. Although STATs have been proposed as important transcription factors for induction of the genes encoding the Pim kinases (310). It would thus have been interesting to study the regulation of Pim kinases in CD8+ T cells using the system described in this thesis although time constraints limited this possibility and other difficulties may have occurred because of limited access to reliable antibodies, inhibitors and transgenic mice. However, further biochemical and translational studies are needed to define expression patterns of Pim kinases in naïve and antigen stimulated T cells, especially in response to γc cytokines. For example are the Pim kinases true down stream targets of IL-2/15 receptors?

Further experiments with Pim knockout mice in an infection model may also highlight whether these mice can mount normal immune responses, focusing on homing patterns and generation of effector memory CTL. This analysis maybe informative as to whether the Pim kinases play an important role in the functional differentiation of peripheral CD8+ T cells.

One other issue that would have been interesting to explore is the identity of the PI3K isoforms relevant for IL-2 and IL-15 signalling. In particular there is always a concern about making conclusions about signalling pathways based solely on the use of pharmacological inhibitors. As discussed in the introduction, T cells express multiple isoforms of PI3K and which ones involved in γc signal transduction is not known. Okkenhaug and colleagues generated mice expressing a catalytically inactive form of the p110δ subunit (p110$\delta^{D910A/D910A}$) referred to as p110δ knock-in mice (81). In the last few weeks of the thesis we were able to carry out one experiment on T cells isolated from these mice. We observed that the ability of p110$\delta^{D910A/D910A}$ T cells to proliferate and grow in response to TCR triggering was impaired but these defects could be overcome by addition of IL-2 or IL-15. These results argue that p110δ is important for TCR but not IL-2/IL-15 signalling. However, one intriguing observation was that IL-2 induced proliferation of p110$\delta^{D910A/D910A}$ T cells was much more sensitive to rapamycin than normal in the same way that Pim kinase deficient T cells show enhanced rapamycin sensitivity. These studies need much more work and were not completed due to time constraints.

Chapter 6

IL-2 and IL-15 differentially regulate PDK1 signal transduction.

6.1 Introduction

Previous data indicated that IL-2 and IL-15 are equal mitogens but are not equal in their ability to regulate cell growth, or protein synthesis. There was also an indication that PI3K may play an important role in IL-2 and IL-15 regulation of cell size. Hence the aim of this chapter was to explore if differences in the effects of IL-2 and IL-15 on PI3K signals could explain the differential effects of these two cytokines on T cell growth. Key PI3K effectors for the regulation of cell growth: are the serine/threonine kinases Phospholipid Dependent Kinase 1 (PDK1) and Protein Kinase B/Akt (389, 410, 436, 573). PDK1 (phosphoinositide dependent kinase-1), was first identified in the context of PI3K-PKB signalling and acts downstream of PI3K to activate PKB (393). Deletion of the PDK1 gene by homologous recombination technology in mice causes embryo lethality (409). Moreover, conditional gene deletion of PDK1 blocks T cell differentiation in the thymus, and reduced PDK1 expression was shown to allow T cell differentiation but block thymocyte proliferation and expansion. Of relevance to the present study is that pre-T cells lacking expression of PDK1 are reduced in cell size

compared to normal pre-T cells revealing for the first time, that PDK1 plays a regulating T cell size and growth (410).

In studies of PDK1 deficient mice a method for the assessment of PI3K/PDK1 signalling in T cells was described. This method quantifies the phosphorylation of the ribosomal S6 subunit on serine235/236 by using flow cytometric analysis of intracellular staining with specific phospho-S6 antisera (410). S6 phosphorylation on ser235/236 is mediated by S6K1 (p70S6K) and occurs down stream of PDK1 via two pathways. Firstly, PDK1 directly phosphorylates p70S6K in its active site or 'T loop' site (574). Secondly, p70S6K is also regulated by the PDK1 substrate PKB via a TSC1/TSC2/mTOR pathway (**Fig 6.2.1**). Importantly, previous studies have indicated that the activation of PI3K is both necessary and sufficient for the activation of PKB and p70S6K in T cells (389) indicating that quantification of S6 phosphorylation will report PI3K activity in T cells. As well, the ability of S6 phosphorylation to monitor PDK1 function has been verified by analysis of PDK1 null T cells (410).

The aim of this chapter was to use S6 phosphorylation as a sensitive and quantitative assay capable of measuring PDK1 signalling pathway activity to look at physiological regulation of these signals in antigen activated T cells responding to cytokines IL-2 and IL-15. Flow cytometric analysis of phosphorylated S6 ribosomal protein allows analysis at a single cell level of the phosphorylation of a downstream target of PI3K/PDK1/PKB/p70S6K (**Fig 6.2.2**). This is a sensitive assay for PDK1 function because it looks at phosphorylation of a downstream target of the enzyme at a point where there has been considerable signal amplification. Moreover, the specificity of the assay can be readily verified as treatment of cells with rapamycin, which inhibits the activity of

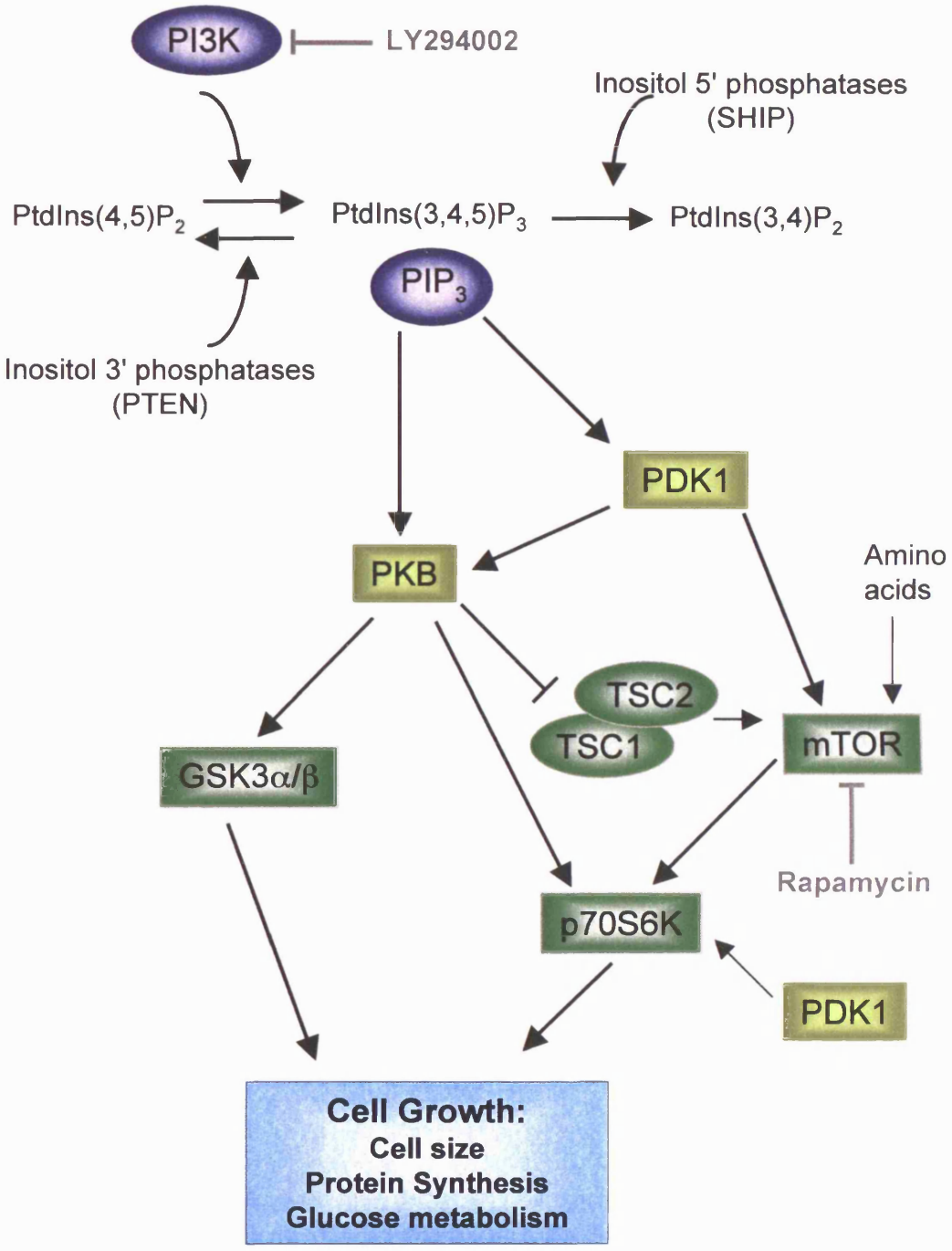


Figure 6.2.1: Signalling pathways contributing to T cell growth. PI3K activity generates $PI(3,4,5)P_3$ which signals to effector molecules PKB , mTOR, p70S6K and regulates glucose metabolism and protein synthesis. PI3K and mTOR activity is inhibited by LY294002 and rapamycin respectively.

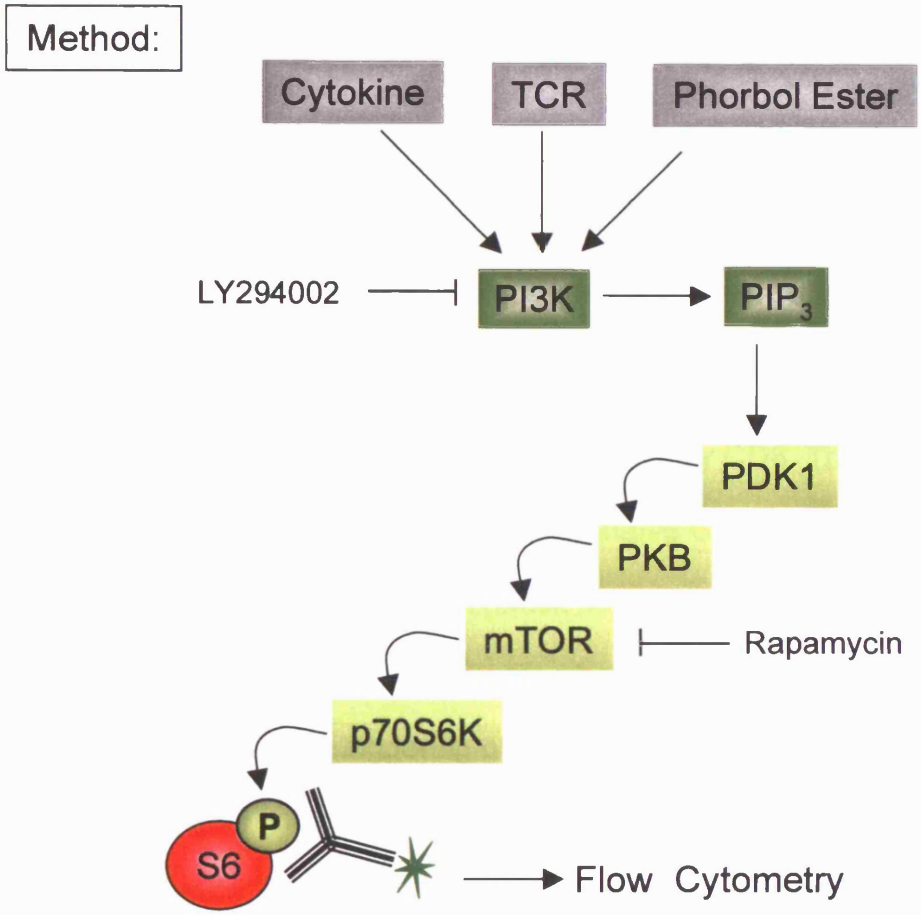


Figure 6.2.2: Measuring PI3K/PDK1 activity by monitoring phosphorylation of effector molecule S6 ribosomal protein. S6 phosphorylation is amplified down stream of PI3K/PDK1 signalling. Biochemical analysis of phosphorylated S6 using flow cytometry allows for the detection of phosphoylation events at a single cell level and proves to be a sensitive and quantitative assay of PI3K/PDK1 activity.

mTOR, rapidly reverses S6 phosphorylation (78, 575). This makes it possible to provide an internal negative control as a standard for each sample. It is also possible to use a pharmacological stimulant, phorbol ester phorbol 12.13 dibutyrate (PdBu) as a positive control to assess the maximum potential for S6 phosphorylation in each cell.

6.2 Results

6.2.1 Antigen activation of naïve T cells dramatically increases phosphorylation of S6 ribosomal protein.

Lymph nodes were taken from P14 LCMV mice and labelled for CD8, Thy1.2 and intracellular phospho-S6 protein. Histograms show naïve CD8+ T cells have very low concentrations of phospho-S6 protein (black filled area), comparable to the rapamycin treated cells (thick grey line) indicating basal levels of phospho-S6 (**Fig 6.2.3a**). Data also show that triggering of the antigen receptor complex with CD3 antibodies (light grey line) or a pharmacological stimulant, phorbol ester phorbol 12.13 dibutyrate (PdBu) (thin grey line) for 30mins induces S6 phosphorylation. The data in **Fig 6.2.3b** compares S6 phosphorylation in naïve T cells to P14 TCR transgenic T cells activated with gp33 specific peptide in the context of MHC I molecules for 48hours. Antigen activation also induces high levels of phospho-S6 protein (black filled), which is comparable to levels induced by phorbol ester stimulation (thin grey line) suggesting antigen stimulation induces the phosphorylation of nearly all S6 protein present in the T cell (**Fig 6.2.3b**).

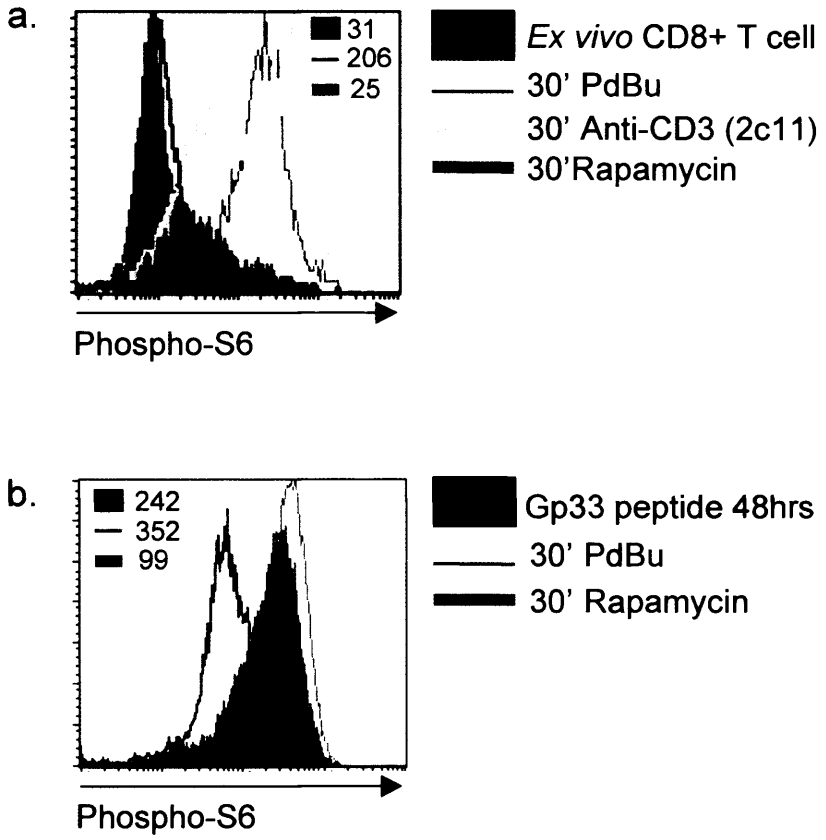


Figure 6.2.3: Phosphorylated S6 protein levels are low in naïve *ex vivo* CD8+ T cells but rapidly increase when stimulated with antigen in the context of MHC class I. FACS histograms indicating relative levels of phosphorylated S6 ribosomal protein. a) *Ex vivo* lymph node P14 naïve CD8+ T cells. b) Antigen activated P14 splenic T cells. Controls: phorbol ester phorbol 12.13 dibutyrate (PdBu), anti-CD3 (2c11) (positive control), 20nM rapamyicn (negative control) for 30mins (30'). All histograms represent CD8a+, Thy1.2+ T cells.

6.2.2 In the absence of exogenous stimuli, antigen activated T cells rapidly lose phosphorylation of S6 protein.

Spleens were taken from P14 LCMV mice and antigen stimulated for two days with gp33 LCMV specific peptide. **Fig 6.2.4a** shows FACS histograms of antigen stimulated CD8+ T cells, which were washed free of exogenous antigen and cultured in medium only without cytokines. A kinetic analysis of these cells was undertaken over 6, 12, 24hours in culture. These data show that antigen activated T cells rapidly lose S6 phosphorylation when cultured without cytokines. This loss of S6 phosphorylation is seen after as little as 6hours without external stimuli (**Fig 6.2.4a**). Data in **Fig 6.2.4b** compares S6 phosphorylation in antigen activated T cells cultured in medium alone or IL-2 over a 24hour period. T cells lose S6 phosphorylation when cultured in medium but are capable of maintaining high levels of S6 phosphorylation when cultured in the presence of IL-2 for the same time period (**Fig 6.2.4b**). These data show that antigen activated CD8+ T cells cannot autonomously maintain the phosphorylation of S6 protein without the constant presence of external stimuli, be it antigen or cytokine growth factor.

6.2.3 PI3K plays a role in regulating IL-2 induction of S6 phosphorylation

Previous studies have shown that the activation of PI3K is both necessary and sufficient for the activation of PKB and p70S6K in T cells (389). To explore whether S6 phosphorylation is an assay for PI3K signalling in T cells we examined the impact of a PI3K inhibitor LY294002 on S6 phosphorylation.

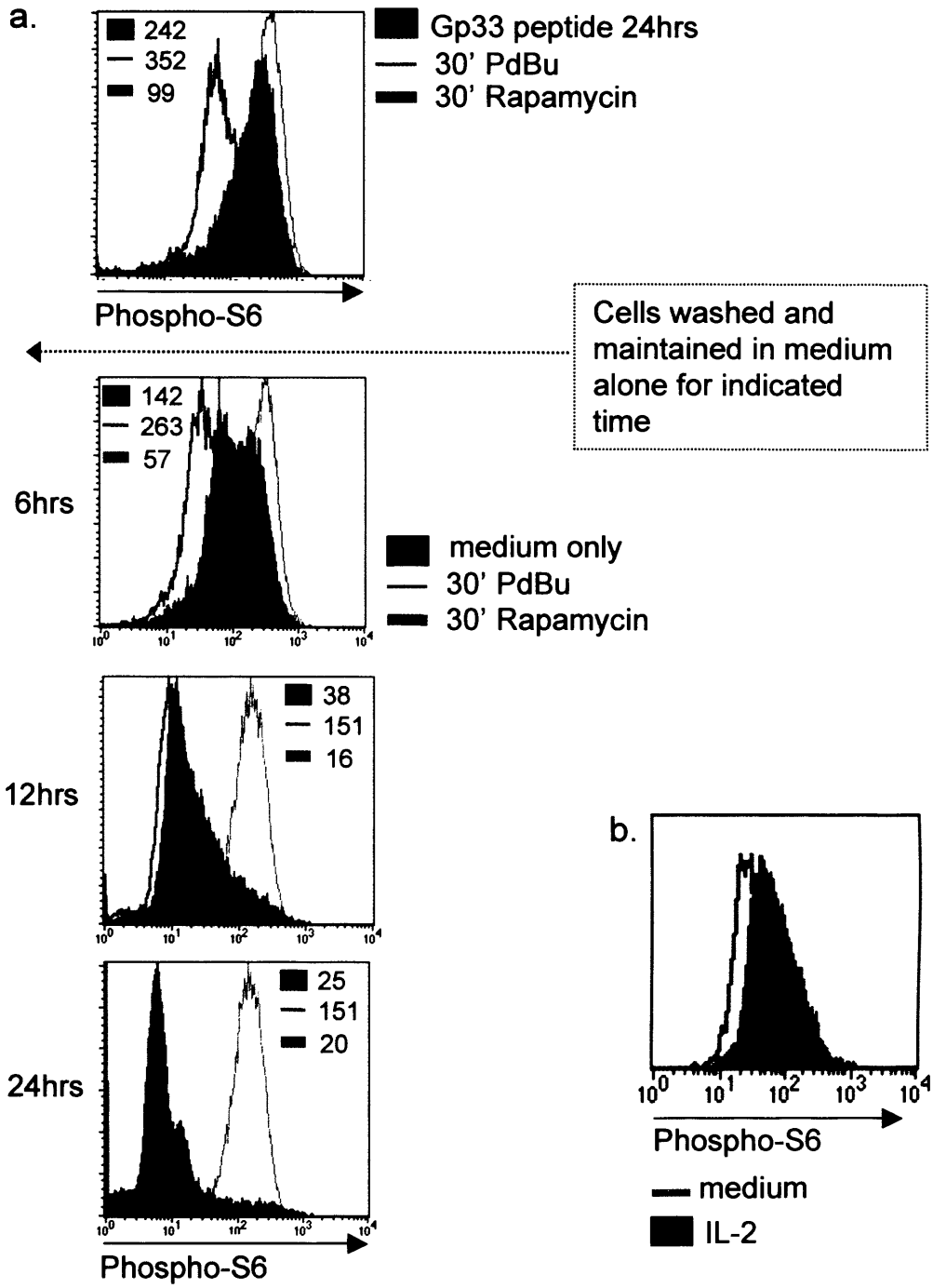


Figure 6.2.4: Activated T cells down regulate S6 phosphorylation when antigen is removed, but maintain phospho-S6 levels in the presence of IL-2. FACS histograms show levels of phosho-S6 in antigen activated P14 LCMV CD8+ T cells a) washed and cultured without exogenous growth factors for 6, 12 and 24 hours. b) Antigen activated CD8+ T cells cultured in medium alone or 20ng/mL IL-2 for 24hours. Controls include 30 minute treatment with phorbol ester (thin grey line) or rapamycin (thick grey line). All histograms represent CD8a+, Thy1.2+ T cells.

Histograms in **Fig 6.2.5** show antigen activated T cells cultured in medium alone (left column), 20ng/mL IL-2 plus or minus 10μM LY294002 (the PI3K inhibitor) for 48hours in total. A kinetic analysis of S6 phosphorylation was undertaken where T cells were stained for intracellular phospho-S6 after 6, 24 and 48hours of cytokine/inhibitor treatment. **Fig 6.2.5** shows phospho-S6 levels rapidly decrease when cells were cultured in medium alone, but cells cultured in IL-2 maintained high levels of phospho-S6 for the entire 48hours. This IL-2 response was lost when cells were co-cultured with IL-2 and the PI3K inhibitor LY294002. Thus the ability of IL-2 to induce phosphorylation of S6 ribosomal protein requires PI3K activity.

6.2.4 A comparison of IL-2 and IL-15 induced S6 phosphorylation

The previous data show that antigen activated CD8+ T cells have high levels of phospho-S6 that is lost if cells are removed from antigen and cultured in medium alone. Phospho-S6 levels are maintained when antigen activated T cells are cultured with IL-2 and this response is dependent on activation of PI3K as co-culture of T cells with IL-2 and the PI3K inhibitor LY294002 abrogates S6 phosphorylation.

Next we compared the ability of IL-2 and IL-15 to regulate the phospho-S6 pathway. At early time points, up to 6hours, the ability of IL-2 and IL-15 to sustain S6 phosphorylation was very similar (**Fig 6.2.6**). However, during prolonged exposure to cytokine only IL-2 could maintain high levels of phospho-S6 whereas the IL-15 response was much lower (**Fig 6.2.6**). Hence T cells maintained in IL-15 for 12-24 hours have markedly lower levels of phospho-S6

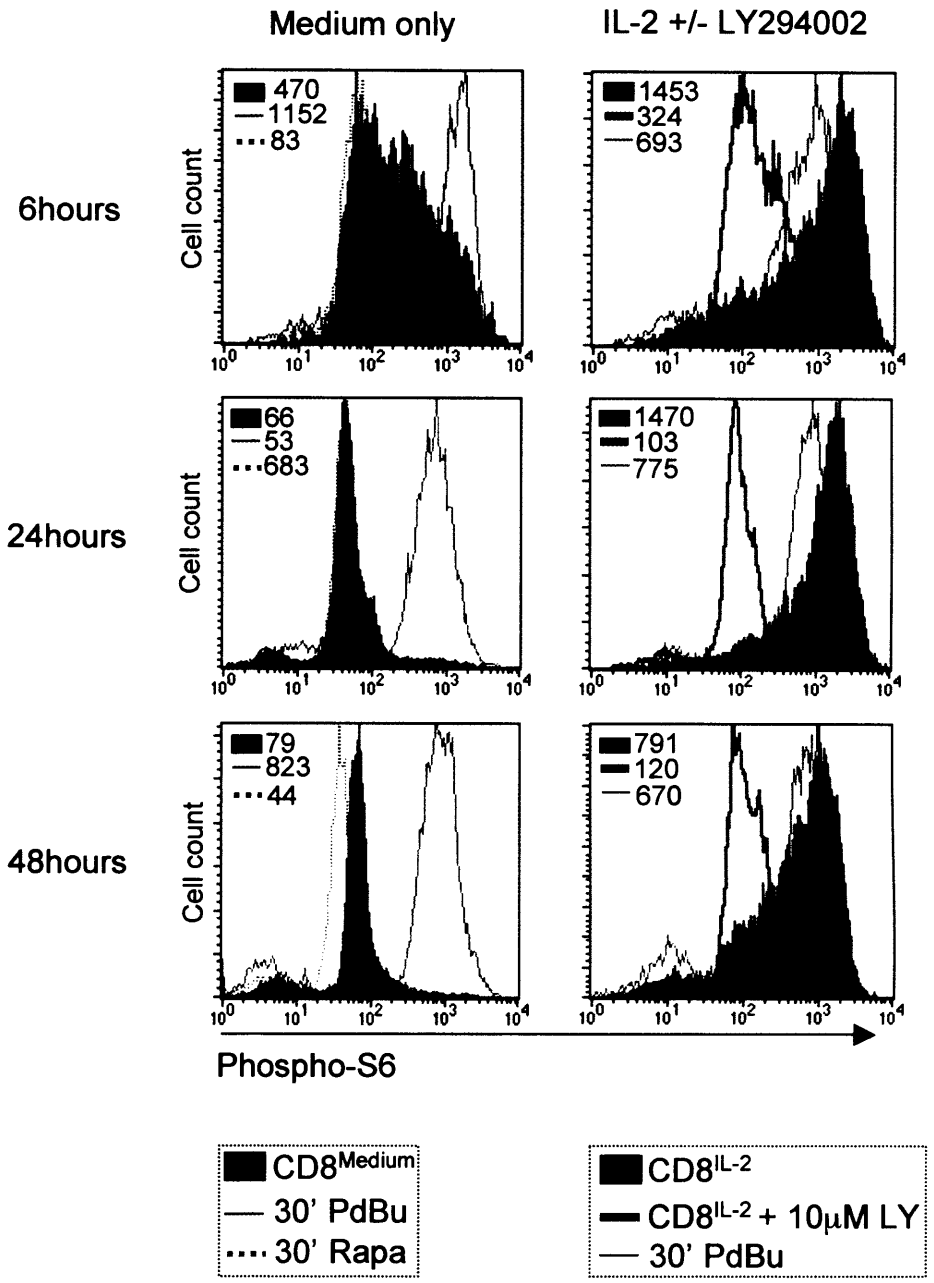


Figure 6.2.5: PI3K plays a role in regulating phospho-S6 levels in $CD8^{IL-2}$ T cells. FACS histograms of antigen activated P14 CD8+ T cells cultured in 20ng/mL IL-2 with or without 10μM LY294002 a PI3K inhibitor for 6, 24 and 48hrs. Controls: 30min (30') treatment with PdBu or 20nM rapamycin previous to staining. All histograms represent CD8a+, Thy1.2+ T cells.

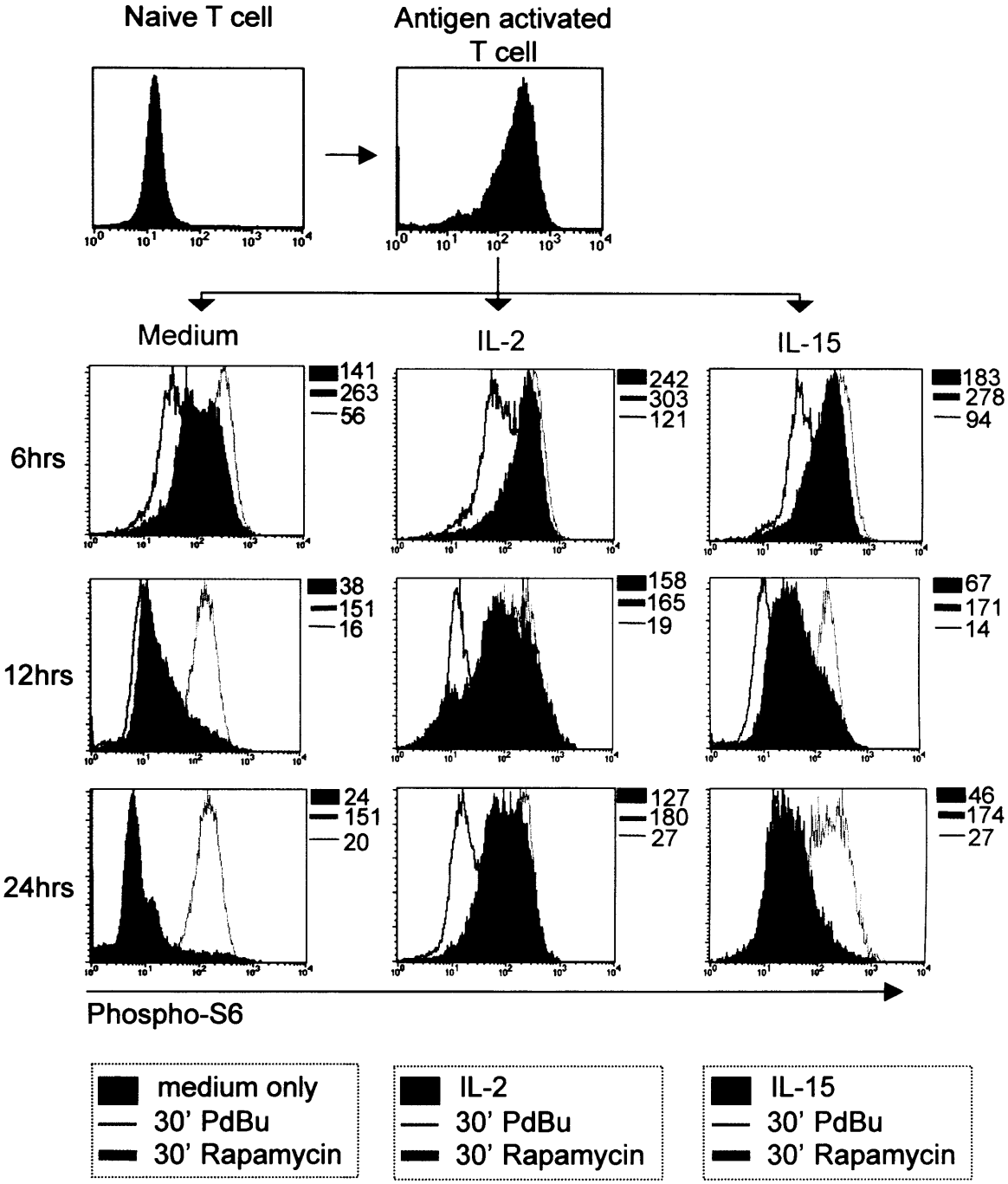


Figure 6.2.6: Antigen activated CD8+ T cells cultured in IL-2 or IL-15 regulate S6 phosphorylation with different kinetics. IL-2 sustains S6 phosphorylation where IL-15 provides a more transient regulation. FACS histograms show expression levels of phosphorylated S6 protein in naïve T cells, antigen activated CD8+ T cells cultured with 20ng/mL IL-2, IL-15 or medium only. Controls: 30min (30') treatment with PdBu or 20nM rapamycin previous to staining. All histograms represent CD8a+, Thy1.2+ T cells.

than cells maintained in IL-2. It should be emphasized that phospho-S6 levels in T cells cultured in IL-15 are higher than those seen in cells cultured in medium lone but considerably lower than cells cultured in IL-2 (**Fig 6.2.6**). It was possible that the difference between IL-2 and IL-15 in terms of their ability to regulate phosphorylation of S6 was due to the fact that CD8+ T cells maintained in IL-15 had lower levels of the substrate S6 protein than cells grown in IL-2. However, phorbol ester (PdBu) treated controls show comparable levels of phospho-S6 protein (**Fig 6.2.6**). Hence T cells maintained in IL-15 or IL-2 have the potential to activate similar levels of S6 phosphorylation if activated pharmacologically but IL-15 is unable to maximise this potential and can only transiently maintain high levels of S6 phosphorylation. In contrast, IL-2 can sustain high levels of S6 phosphorylation for a prolonged period.

The ability of IL-2 to regulate S6 phosphorylation is dependent on PI3K. The experiment in **Fig 6.2.7** assesses the role of PI3K in IL-15 induction of S6 phosphorylation. At early time points S6 phosphorylation is high in the presence of 20ng/mL of IL-15. These levels are reduced over the same 6hour time period by the presence of 10μM LY294002, which are comparable to levels seen in medium only control cells. The data show that T cells co-cultured with IL-15 and LY294002 cannot sustain S6 phosphorylation compared to cells cultured in cytokine alone. IL-15 phosphorylation of S6 is thus mediated by PI3K. Accordingly, differences between IL-2 and IL-15 in terms of the kinetics of their S6 response is an indication that there are quantitative and kinetic differences in the activation of PI3K by these two cytokines.

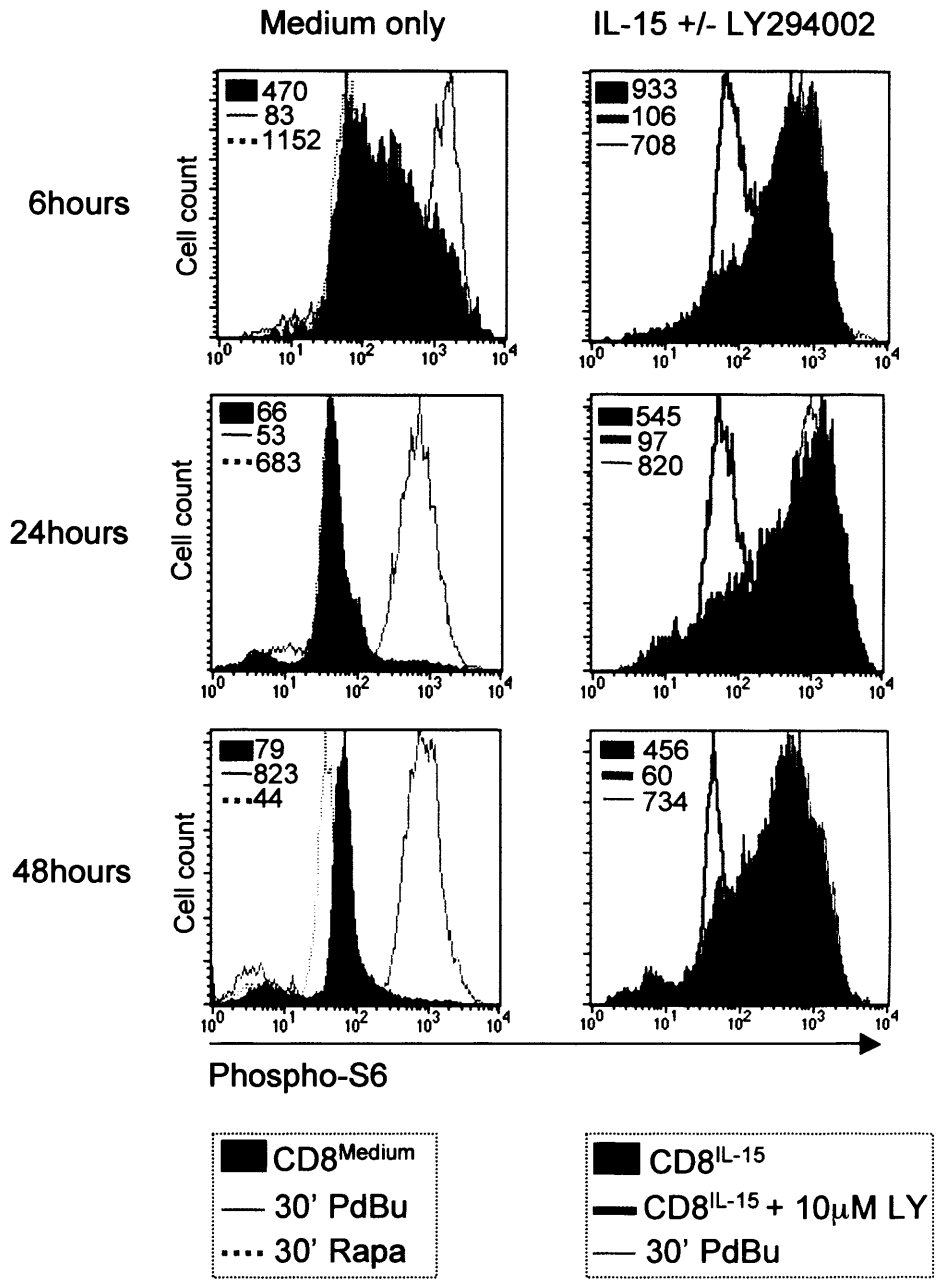


Figure 6.2.7: PI3K plays a role in regulating phospho-S6 levels in CD8^{IL-15} T cells. FACS histograms of antigen activated P14 CD8+ T cells cultured in 20ng/mL IL-15 with or without 10μM LY294002 a PI3K inhibitor for 6, 24 and 48hrs. Controls: 30min (30') treatment with PdBu or 20nM rapamycin previous to staining. All histograms represent CD8a+, Thy1.2+ T cells.

6.2.5Regulation of IL-2 and IL-15 receptors

Differences in the kinetics of IL-2 and IL-15 induction of phospho-S6 reveals that IL-15 initiates more transient signalling pathways in CD8+ T cells than IL-2. A predominant mechanism used by T cells to terminate cytokine signalling is to regulate surface expression of cytokine receptors (106, 124, 539). The high affinity binding of IL-2 is mediated by a complex of; the common cytokine receptor γ chain (γc), CD122 (the IL-2 receptor β chain) and CD25 (the unique IL-2 receptor α chain). IL-15 interacts with cells via binding to the unique IL-15 receptor α subunit (IL-15Rα), which presents IL-15 in *trans* to γc and β (CD122) subunits present on nearby cells (207, 210). IL-2 binding to its receptor triggers receptor internalisation and ligand degradation but simultaneously up regulates expression of CD25 (576), thereby preventing receptor down regulation and allowing activated T cells to sustain proliferative and growth responses to IL-2. In contrast, exposure to IL-15 is reported to down regulate expression of high affinity IL-15 receptors with a resultant loss of IL-15 responsiveness (577).

To examine the possibility that IL-15 down regulates expression of the IL-15 receptor α subunit in antigen primed CD8+ mouse T cells, flow cytometry was used to quantify IL-15 and IL-2 receptor subunits on the surface of antigen activated T cells maintained in medium alone or in the presence of 20ng/mL IL-2 or IL-15 (**Fig 6.2.8a & b**). Surface levels of CD25, the unique IL-2 receptor α chain, are markedly up regulated when antigen activated CD8+ T cells are grown in IL-2 versus media alone (**Fig 6.2.8a**). IL-15 is not as potent as IL-2 at maintaining CD25 levels but still stimulates CD25 expression when compared to media alone. Activated CD8+ T cells also express IL-15Rα but surface levels of

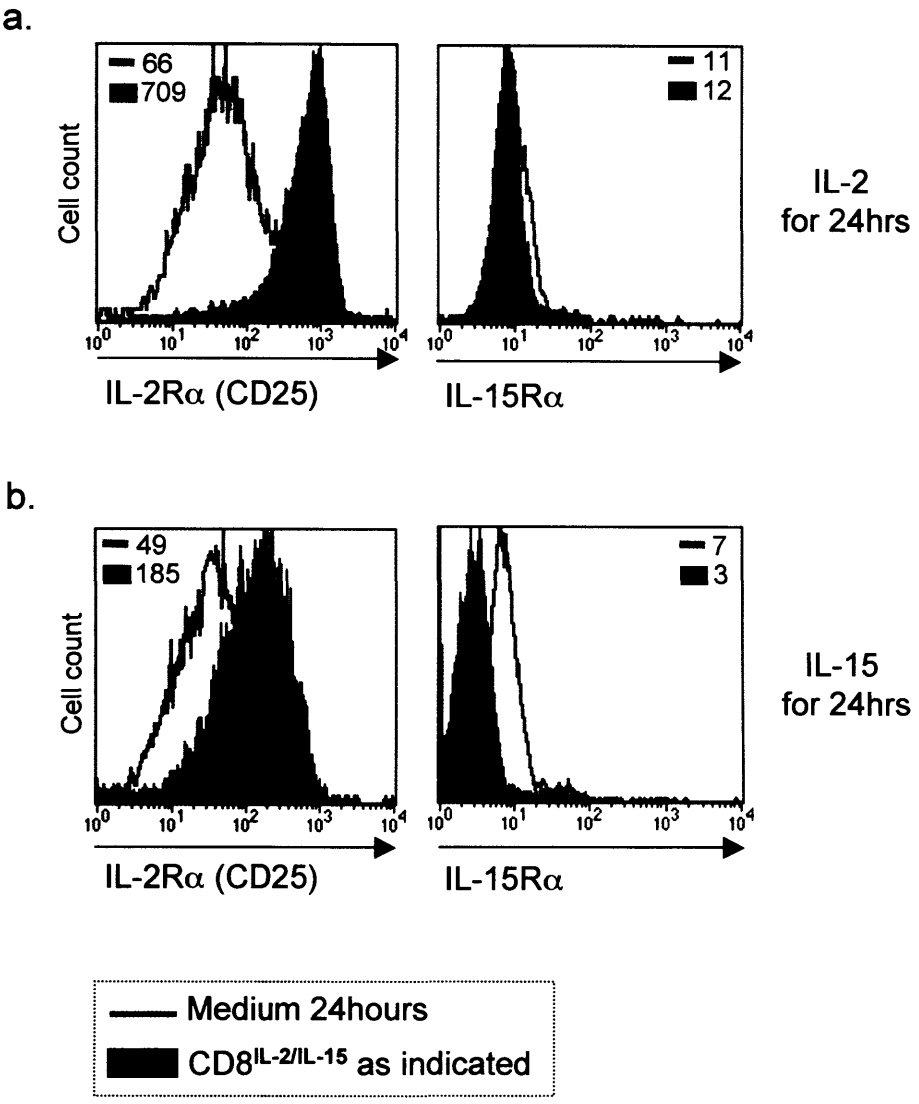


Figure 6.2.8: Transient signalling through PI3K pathways maybe a result of cytokine receptor down regulation. FACS histograms showing expression levels of IL-2Rα (CD25) or IL-15Rα subunits on antigen primed P14 CD8+T cells cultured in a) 20ng/mL IL-2 ($CD8^{IL-2}$) and b) 20ng/mL IL-15 ($CD8^{IL-15}$) or medium alone (overlayed) for 24 hours. R: receptor

this receptor are not maintained in the presence of IL-15 (**Fig 6.2.8b**). IL-15Rα chain expression is relatively high in antigen activated T cells cultured in media alone or in IL-2 but relatively low in cells stimulated with IL-15. Hence, IL-2 and IL-15 differ in the way they regulate expression of their receptors: IL-2 triggers a positive feedback mechanism that up regulates expression of the IL-2 receptor α subunit: the key receptor subunit for high affinity IL-2 receptor expression. Conversely, levels of IL-15 receptor α chain decline in CD8+ T cells cultured with IL-15.

6.3 Discussion

Data in this chapter shows that antigen activation of *ex vivo* CD8+ T cells is a very strong stimulus for the induction of S6 phosphorylation. When the antigen stimulus is taken away and the cells cultured in medium alone the ability of the cell to phosphorylate S6 protein is compromised and levels of phospho-S6 are rapidly down regulated. Thus, antigen activated T cells cannot autonomously maintain S6 phosphorylation. Further analysis showed that antigen activated T cells exposed to growth factors IL-2 and IL-15 are able to maintain S6 phosphorylation via a PI3K mediated pathway, although with different kinetics and magnitude. Initially the IL-2 and IL-15 response are equivalent in magnitude but there is a significant difference in the prolonged response to these two cytokines. IL-2 has the capacity to sustain S6 phosphorylation at high levels for several days whereas IL-15 only transiently induces high levels of S6 phosphorylation and very low levels during a prolonged response. The major difference between IL-2 and IL-15 signalling is thus temporal and quantitative: IL-15 signalling is relatively transient whereas IL-2 signalling is sustained.

The phosphorylation of S6 ribosomal protein is a result of signalling through the PI3K/PDK1/PKB/p70S6K pathways (78, 389, 410, 517, 578, 579). Differences between IL-2 and IL-15 are thus consistent with differences in the ability of these cytokines to sustain PI3K activity. To test this hypothesis directly would require quantitative analysis of cellular levels of PtdIns(3,4,5)P_3 (PIP_3) in antigen activated T cells cultured in IL-2 or IL-15. Measuring PIP_3 levels can be achieved by labelling cells with [^{3}H]inositol or [^{32}P]orthophosphate followed by lipid extraction and analysis by HPLC. However, cell labelling with inositol is not very efficient, where labelling to isotopic equilibrium can take days and can be inconvenient, requiring large amounts of radioactivity. Both labelling procedures also suffer from the drawback of elaborate and time-consuming HPLC analysis and require very large numbers of cells. Another method for the measurement of PIP_3 mass in cells uses a radio-ligand displacement assay that does not require cells to be metabolically labeled but does require large numbers of cells (580). Another approach for measuring PI3K activity is to measure phosphorylation events known to be down stream of PI(3,4,5)P_3 such as the phosphorylation of Akt/PKB or the activation/phosphorylation of p70S6K. Conventional western blotting using phospho-specific antibodies and ECL technology is the most common approach to look at signalling pathways and can be a very sensitive assay. However, from the present data it is shown by flow cytometric analysis that there is only a two-fold difference in signalling between IL-2 and IL-15 and ECL detection is insufficiently quantitative in this range. Hence, the use of phospho-specific antibodies in intracellular staining for flow cytometry is an attractive option, as this method is fast and provides strong quantitative data. However, the use of phospho-specific antibodies in flow cytometry can be

difficult and relies on the integrity and specificity of the antibodies used. We would have liked to have used flow cytometry to quantify the phosphorylation of signalling molecules in the IL-2/IL-15 receptor signalling cascade including PKB/Akt, the MAP kinases p42/44 ERK1//2 and the STATs, STAT3 and STAT5. MAP kinase phosphorylation by cytokines is very weak and transient and difficult to quantify accurately when relying on western blotting. As well, our attempts to use flow cytometric analysis to look at MAP kinase phosphorylation failed, probably due to the low stoichiometry of MAP kinase phosphorylation per cell. We also failed to get any convincing data with phosphoSTAT5 antibodies. A sensitive and quantitative method of measuring STAT activity involves DNA pull down experiments where active STATs would be sequestered by target promoter oligonucleotide sequences and assayed by western blot analysis with STAT5 antibodies. These experiments were not performed due to time constraints of the thesis. Even though we didn't directly measure STAT5 activation, it is known that STAT5 mediates IL-2 up regulation of CD25 (581). Data from previous chapters indicate that antigen activated cells cultured in IL-2 or IL-15 differentially regulate expression levels of CD25 over a 24hour period which would be consistent with temporal and quantitative differential regulation of STAT5 phosphorylation in response to IL-2 and IL-15 in antigen activated CD8+ T cells.

So, why is IL-15 activation of S6 phosphorylation transient whereas the IL-2 response is sustained? The possibility of IL-15 up regulating a global negative regulator pathway that antagonizes PI3K signals (e.g. an inositol lipid phosphatases such as PTEN or SHIP) can be excluded mainly because of the fact that T cells maintained in IL-15 can immediately up regulate PI3K pathways

and increase cell size if re-exposed to IL-2. However there are other regulators of cytokine signalling namely the SOCS proteins that have recently been suggested to elicit a more specified inhibitory function for IL-15 versus IL-2 signalling. The SOCS family are cytokine induced adaptor proteins that function as negative feedback inhibitors of cytokine receptor signalling by inhibiting the JAK-STAT signal transduction pathway. Over the past few years it has become obvious that the 7 members of the SOCS family all play important and specific roles in many different cell types (582, 583). Specifically SOCS1 has been found to play an important role in T cell homeostatic proliferation and is constitutively expressed in CD8+ T cells. Rottapel and colleagues have argued that loss of SOCS1 preferentially regulates IL-15 versus IL-2 responses where they show that SOSC1-/- T cells with a memory phenotype (CD44+ T cells) were hyper responsive to IL-15 (584). Further more, Ilangumaran et al have suggest that SOCS1 is an indispensable attenuator of IL-15 receptor signalling in thymocytes, where IL-15/IL-15R signalling induced sustained STAT5 phosphorylation and maintained massive cell proliferation of SOCS1-/- CD8+ thymocytes (585). However, Alexander and colleagues have shown that SOCS1 also regulates IL-2 signaling such that SOCS1 null mice lose tolerance and develop autoimmune disease (586).

Thus, it is unclear that IL-2 and IL-15 differ in their ability to regulate expression of SOCS proteins but in this context it is noteworthy that SOCS proteins have recently been found to interact with ubiquitin ligase complexes, and have been shown to target cytokine receptors and associated tyrosine kinases for proteosomal degradation (587, 588). Thus we might speculate that SOCS1 could target IL-15Rα chain for degradation limiting the signalling capacity of IL-

15 down stream to PI3K and other effectors. However, there are other mechanisms by which SOCS proteins can be inhibitory including direct inhibitory effects on JAK-STAT signalling.

One option we did consider was that the different kinetics of IL-2 and IL15 signalling results from differences in the way these cytokines are presented to T cells. IL-2 binds as a soluble ligand whereas IL-15 is thought to be presented in *trans* by the IL-15 receptor α subunit to β/γ subunits on neighbouring cells (207, 210). The signal strength delivered by IL-15 presented in *trans* may be lower than that delivered by *de novo* binding of IL-2 to its trimeric receptor complex. This possibility cannot be excluded but the simplest explanation for the relative transience of the IL-15/PI3K response compared to the IL-2 sustained response resides in differences in the way these two cytokines can modulate expression of their own receptors. IL-2 thus binds to its high affinity receptor, is internalised and degraded. However, IL-2 signalling is sustained because IL-2 up regulates expression of its α subunit, which replenishes surface levels of high affinity IL-2 receptors that are then available for occupancy by additional IL-2 molecules. Hence during the sustained response to IL-2 there is constant formation of new IL-2/IL-2 receptor complexes. In contrast, IL-15 receptor levels decline during continued exposure to IL-15 thus reducing the number of available IL-15 receptors during sustained exposure to cytokine. In this respect IL-15 receptor regulation is reminiscent of the IL-7 receptor, where IL-7 exposure results in down regulation of IL-7 receptor expression as a mechanism to prevent T cells competing for limiting quantities of cytokine once the appropriate cell response has been stimulated (539).

Could temporal differences in PDK1 signalling translate into differences in protein synthesis? In particular, would the ability of IL-2 to sustain PI3K signal transduction pathways over several days allow this cytokine to sustain T cell protein synthesis at high levels for a prolonged period? The answer is yes as data from the present and previous chapters show that the ability of T cells to maintain protein synthesis is driven by constant signal input as removal of cytokine or inhibition of PI3K with LY294002 almost immediately abrogates amino acid uptake and amino acid incorporation into proteins. The differential actions of IL-2 and IL-15 on T cell growth responses thus correlates well with the differential kinetics of the PI3K signalling patterns stimulated by these two cytokines.

As discussed, PI3K has been shown to regulate glucose metabolism and it is quite likely that IL-2 and IL-15 differentially regulate glucose metabolism. In fact, when activated T cells are grown in the presence of IL-2 and fully complimented medium containing the pH indicator phenol red, the media rapidly changes colour reflecting a lowering of its pH as a consequence of lactic acid production by the T cells. In contrast, T cells maintained in IL-15 do not have this effect on the media indicating that their rate of lactic acid production is not as high. However over this time these cells are still undergoing mitosis at a similar rate illustrating the fact that these cytokines are equivalent mitogens but not equivalent growth factor in context of metabolic capacity.

IL-2 promotes differentiation of effector CTL and IL-15 promotes memory T cell development (40, 524). Could the different biological actions of IL-2 and IL-15 on CD8+ T cell function explain temporally quantitative differences in PI3K

activation by these cytokines? Data that support this theory includes the fact that PI3K signalling is necessary for CTL differentiation (589). As well, low doses of IL-2 mimic IL-15 and induce the differentiation of memory T cells rather than effector CTL (48). The significance of these data are that low doses of IL-2 result in transient signalling because activated T cells consume (i.e. internalise and degrade) IL-2 and at low doses the cytokine is rapidly depleted from the culture medium and hence only transiently available to the T cell population. It is also noteworthy that IL-7 transiently signals to T cells because it down regulates expression of its receptor (539). IL-7 does not mimic the effect of IL-2 on antigen primed T cell mass, and like IL-15 is associated with the development of memory T cells rather than cytolytic effectors (262, 266).

In summary, the present data demonstrates that cytokines that are equivalent mitogens and can have different potency in terms of regulating PDK1 signalling pathways. We predict that the ability of IL-2 to sustain PDK1 signal transduction pathways over several days allows this cytokine to sustain T cell protein synthesis at high levels for a prolonged period.

Chapter 7

γc cytokines differentially regulate cell growth in antigen and anti-CD3 activated T cells.

7.1 Introduction

The common gamma cytokine receptor chain (γc) is a component of the receptors for interleukin 2 (IL-2), IL-4, IL-7, IL-9, IL-15 and IL-21. Mutation in the gene that encodes γc results in X-linked severe combined immunodeficiency (XSCID) in humans and in mice (87, 119, 274, 521). Signals dependent on γc have been shown to be important in the lymphoid compartment particularly for T cells, where they have been shown to play a crucial part in peripheral T cell homeostasis and thymocyte development (87, 119, 274, 521, 590). Data in previous chapters suggests that IL-2 and IL-15 confer differential actions as growth factors whilst maintaining equal potency for mitogenesis. These data presented the possibility that different members of this cytokine family may act in different ways to control the growth and metabolic status of antigen activated T cells.

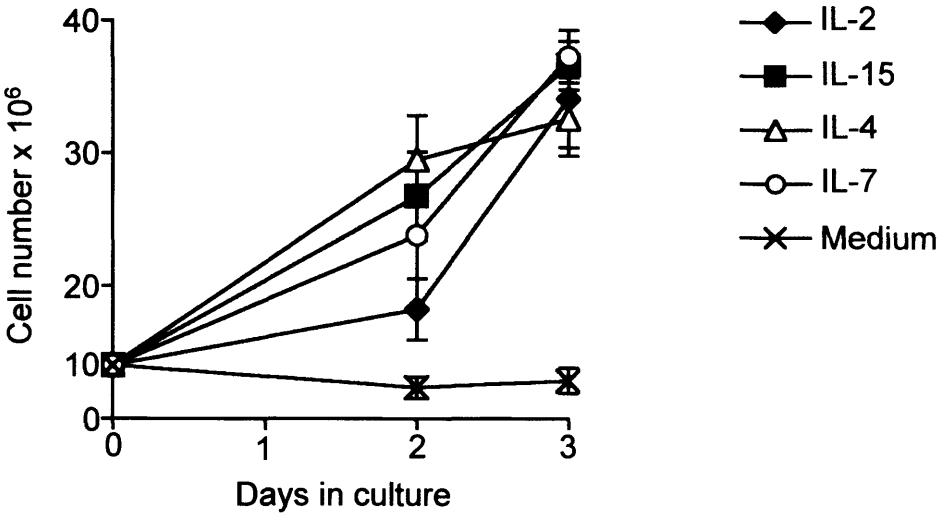
In the following chapter we compare the effects of IL-2, IL-15, IL-7 and IL-4 on T cell growth.

7.2 Results

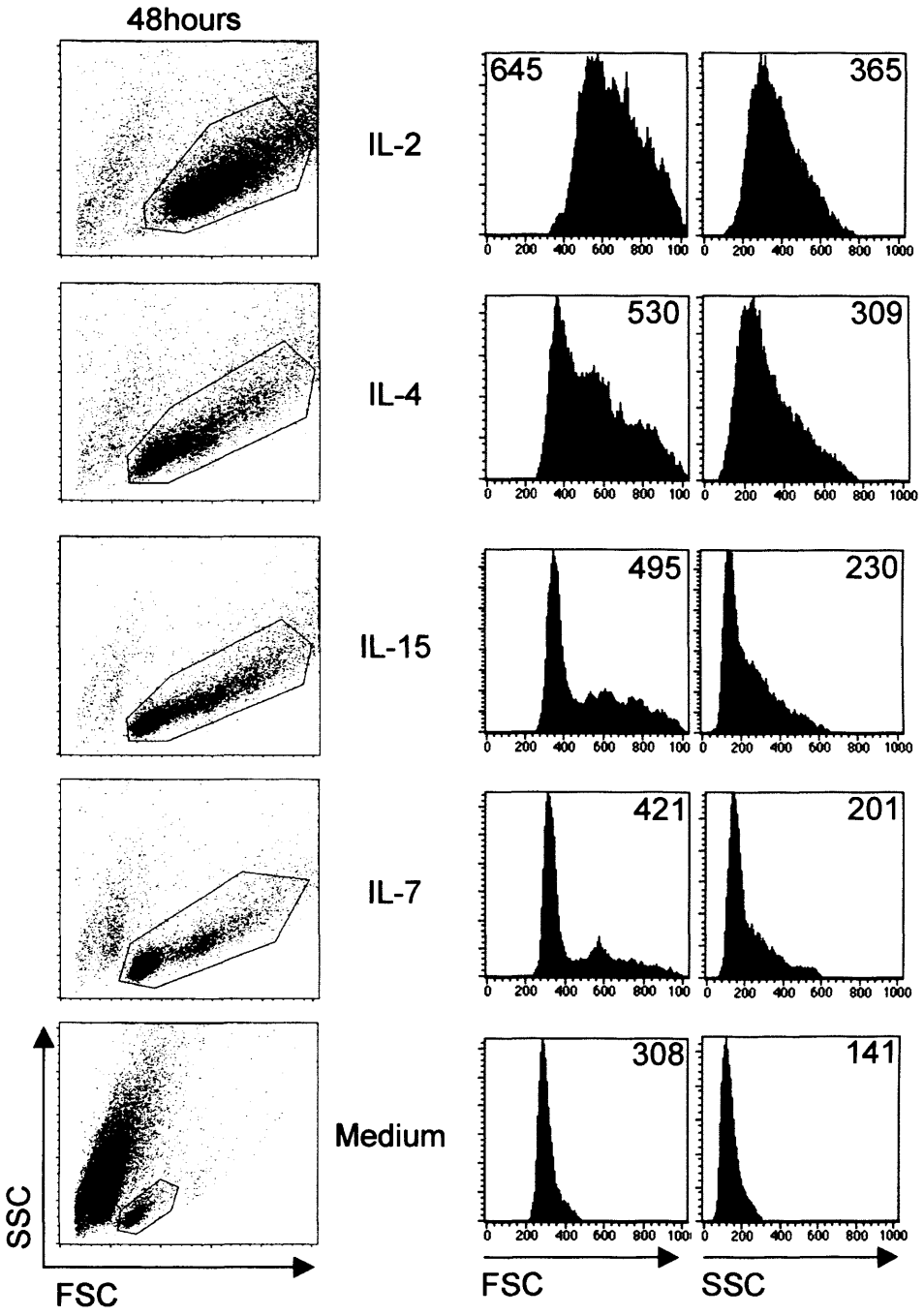
7.2.1 Common gamma chain cytokines differentially regulate the size and protein content of activated CD8+ T cells.

Lymph nodes and spleens were taken from P14 LCMV transgenic and C57BL6J wild type mice, mashed, pooled and activated in vitro. The P14 CD8+ T cells were activated with gp33 specific peptide (1μM) and wild type T cells activated by cross-linking of the TCR using an anti-CD3 monoclonal antibody 2C11 (1μg/mL), both stimuli were carried out for 48hours in culture. Populations of activated T blasts were washed and cultured in the presence of 20ng/mL IL-2, IL-15, IL-7, IL-4 or medium alone. Analysis of T cell growth and proliferation was taken over the first three days of culture and show IL-2, IL-15, IL-7 and IL-4 are similar mitogens for antigen activated T cells (**Figure 7.2.1**).

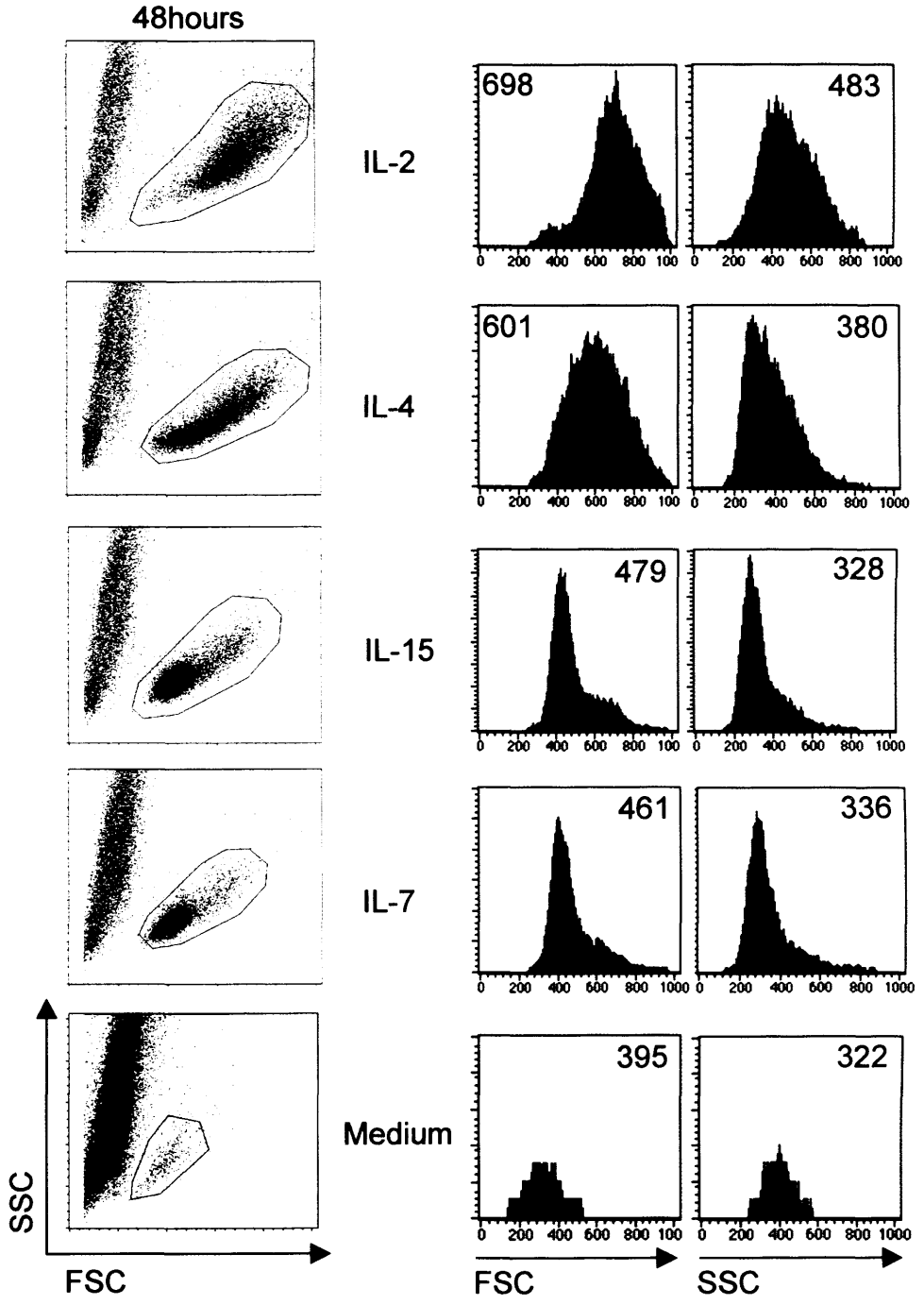
FACS analysis of T cell size was shown as a measure of forward and side light scatter (FSC/SSC) and undertaken on CD3+ gated T cells, where P14 LCMV cultures were additionally gated for CD8+ co-receptor. **Figures 7.2.2** & **7.2.3** show FACS dot plots and histograms of antigen activated P14 CD8+ and wild type T blasts cultured in the presence of γc cytokines as indicated. After 48hours in culture it was clear that IL-2 is the only cytokine capable of maintaining the large granular size indicative of effector T cells in both T blast cultures. T blasts cultured in IL-4 are slightly smaller than IL-2 cultured blasts but larger than cells cultured in either IL-15 or IL-7. Hence, it is clear that IL-4 is more potent than IL-15 and IL-7 at maintaining activated T cell size, however, IL-2 far exceeds all three in it's potential too maintain T cell size.



7.2.1: Cell count of antigen activated T cells cultured in the presence of cytokines IL-2, IL-15, IL-4 and IL-7. Graph shows a cell count taken of antigen activated P14 CD8+ T cells cultured in 20ng/mL of IL-2, IL-4, IL-15, IL-7 and medium alone for 3 days.



7.2.2: Size of antigen activated T cells cultured in the presence of cytokines IL-2, IL-15, IL-4 and IL-7.
FACS dot plots and histograms of P14 CD8+ T cells stimulated with specific antigen for two days, washed and cultured in 20ng/mL of IL-2, IL-4, IL-15, or IL-7 for 48hours.



7.2.3: Size of anti-CD3 stimulated splenic T cells cultured in the presence of IL-2, IL-15, IL-4 and IL-7. C57BL6J splenic T cells were stimulated with 1μg/mL anti-CD3 antibody (2C11) for 48hours, washed and cultured in 20ng/mL of IL-2, IL-4, IL-15, IL-7 and medium only. FACS dot plots and histograms show FSC/SSC analysis of these cells after 48hours in culture.

Protein concentration of anti-CD3 stimulated T cells cultured in IL-2, IL-15, IL-4 and IL-7 was determined. **Figure 7.2.4** shows that cells cultured in IL-2 maintain higher concentrations of protein after 3 days in culture than cells growing in the presence of IL-4, IL-15 or IL-7. This data corroborates with the flow cytometric analysis of T cell size in response to these cytokines and suggests that IL-2 is the only member of the gamma chain cytokine family capable of maintaining a high rate of protein synthesis and large cell size, which contrasts with the fact that all cultures were proliferating or dividing at similar rates, see **Figure 7.2.1**.

7.2.2 Common gamma chain cytokines differentially regulate the phosphorylation of S6 ribosomal protein.

The differential effect of IL-2 and IL-15 on T cell growth correlated with differences in their ability to activate PDK1 mediated signalling pathways as judged by measurements of S6 phosphorylation. Accordingly, we compared the effect of IL-2, IL-4, IL-7 and IL-15 on S6 phosphorylation.

Lymph node and spleen were taken from P14 LCMV and wild type mice, mashed, pooled and stimulated in vitro with 1μM of gp33 specific peptide or 1μg/mL of anti-CD3 antibody, respectively. Activated T blasts were washed and cultured in 20ng/mL of cytokines IL-2, IL-4, IL-15, IL-7 or medium only for negative control. **Figure 7.2.5** and **7.2.6** show FACS histograms indicating phosphorylation levels of S6 ribosomal protein in antigen activated P14 CD8+ T cells and wild type T cell blasts in response to γc cytokines. Antigen activated T cells cannot maintain S6 phosphorylation when cultured in medium alone.

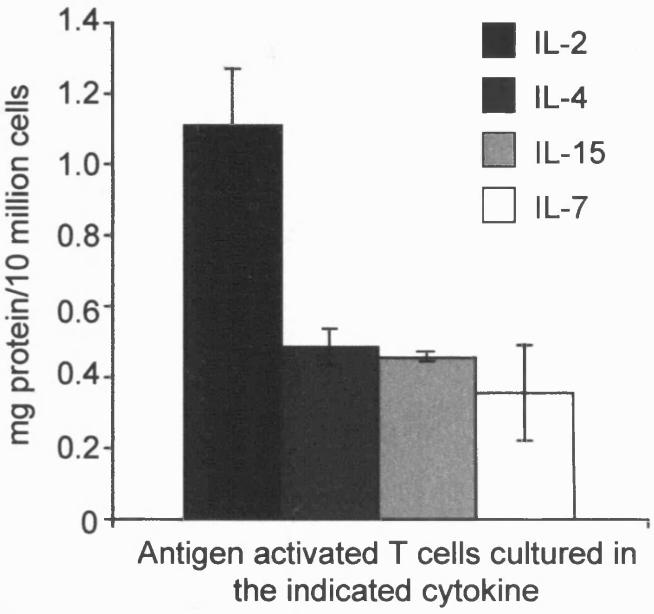
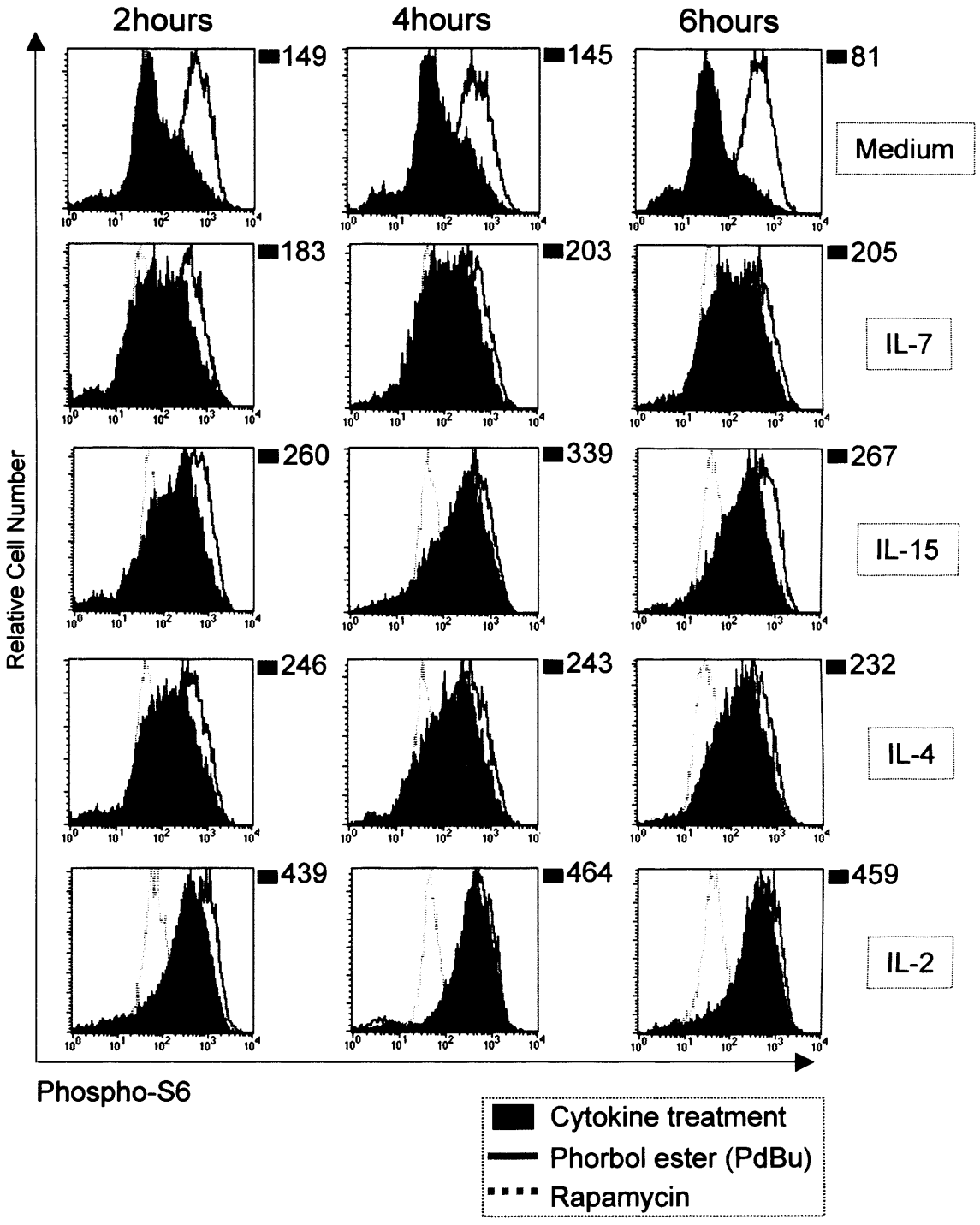
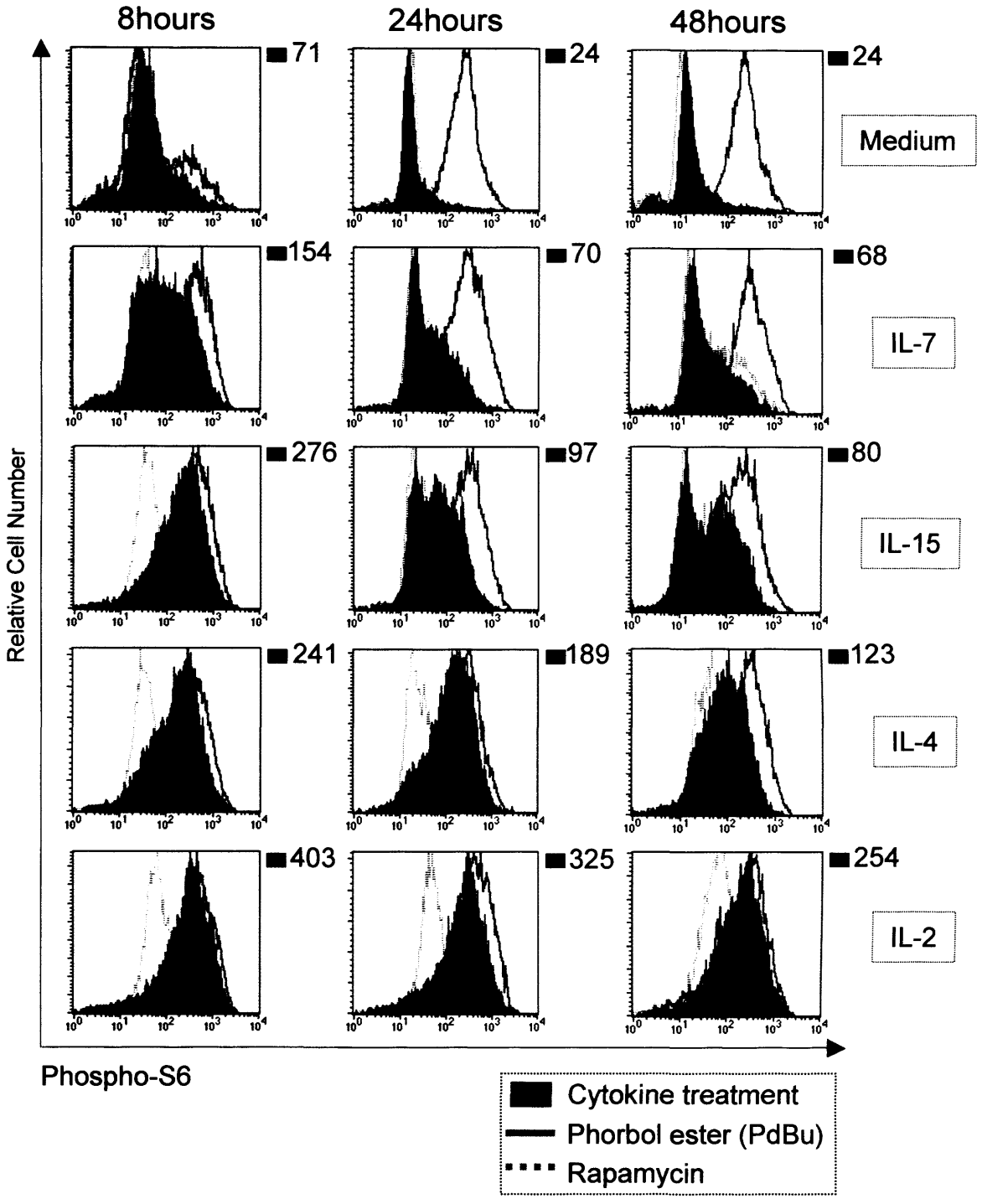
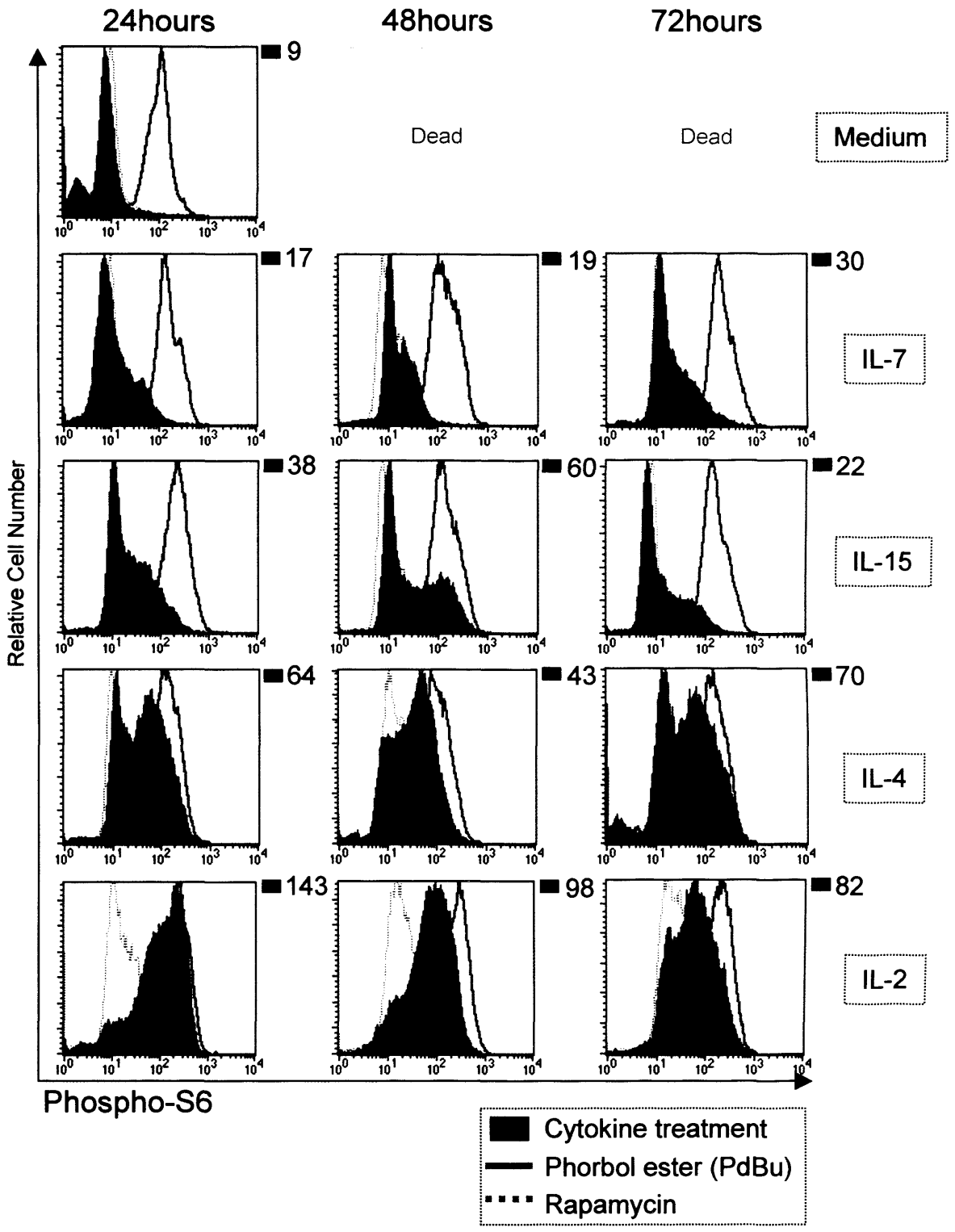


Figure 7.2.4: Concentration of protein maintained by anti-CD3 activated T cells cultured in the presence of cytokines IL-2, IL-15, IL-4 and IL-7 for 72hours. Graph shows C57BL6J splenic T cells stimulated with anti-CD3 antibody (2C11) for 48hours, washed and cultured in 20ng/mL of IL-2, IL-4, IL-15, and IL-7 for 3 days after which cells were lysed and protein concentration determined. Medium control concentrations are not shown due to death of the culture after 48hours.



7.2.5: Phosphorylation of S6 ribosomal protein in antigen activated CD8+ T cells responding to cytokines IL-2, IL-15, IL-4 and IL-7.FACS histograms show antigen activated P14 CD8+ T cells cultured in the presence of 20ng/mL IL-2 , IL-4, IL-7, IL-15 and medium only control. Samples were fixed and stained after 2, 4, 6, 8, 24 and 48hours in culture. Controls: 30min treatment with PdBu and 20nM Rapamycin. All histograms represent CD3+T cells.


8hours
24hours
48hours
71
24
24
Medium
154
70
68
IL-7
276
97
80
IL-15
241
189
123
IL-4
403
325
254
IL-2
Relative Cell Number
Phospho-S6
Cytokine treatment
Phorbol ester (PdBu)
Rapamycin



7.2.6: Phosphorylated S6 protein in anti-CD3 activated wild type T cells cultured in the presence of cytokines IL-2, IL-15, IL-4, IL-7. FACS histograms show levels of phospho-S6 in anti-CD3 stimulated C57BL6J splenic T cells cultured in 20ng/mL of IL-2, IL-4, IL-15, IL-7 and medium only for 24, 48 and 72 hours. Cells cultured in medium died after 24hours. All histograms represent CD3+ T cells.

Here it is most striking that even after 2hours the antigen activated T cells had lower levels of phospho-S6. When comparing the effects of IL-2, IL-15, IL-4 and IL-7, it is clear that IL-2 is the only cytokine capable of maintaining high phospho-S6 levels in activated T cells. IL-4 can maintain some level of phospho-S6 higher than that of IL-15 and IL-7, although not the equivalent of IL-2. IL-7 and IL-15 rapidly lose phospho-S6 levels over a very short period of time, although, the level maintained is higher than T blasts cultured in medium alone.

7.3 Discussion

The data in this chapter suggests that IL-2 is the most potent T cell growth factor when compared to other γc cytokine family members IL-7, IL-15 and IL-4. Moreover IL-2>IL-4>IL-15≅ IL-7>>>medium (no stimulus) in a scale of the most potent growth factor for activated T cells. The importance of this group of cytokines to T cell homeostasis, proliferation and differentiation is well known and there have been many comparative studies of their actions as mitogens, survival factors and differentiation factors. However, little is known about the growth or cell size promoting capacity of the γc cytokines even though an understanding of how these cytokines control cell size and protein synthesis may prove advantageous for predicting their immunotherapeutic value.

The fact that such diversity in the ability to regulate growth occurs may act in defining cytokine function in the immune system. For example, IL-7 and IL-15, that has limited growth factor potential, function as naïve and memory T cell survival factors (40, 591). Hence, these cytokines act to sustain small non-effector cells, which have a low metabolic capacity and maintain very low

bioenergetic levels. Thus, IL-15 and IL-7 do not need to induce high rates of metabolism and protein synthesis to fulfil their role. They just need to sustain T cell survival by the maintenance of mitochondrial potential through the regulation of anti- and pro- apoptotic proteins (434).

The present results also indicated that IL-4 was not as potent a growth factor as IL-2 but was more potent than IL-7 and IL-15. The present results analysed IL-4 responses in a population with a high percentage of CD8+ effector T cells and it would be very interesting to extend analysis of IL-4 growth effects to CD4+ T cells where IL-4 is known to play a central role in the differentiation of antigen-activated CD4+ T helper cells. IL-4 polarizes the differentiation of T cells to the Th2 population that secrete IL-4, IL-5, IL-9, IL-6, and IL-13. Thus one would imagine that Th2 cells would need to maintain relatively high levels of protein synthesis to fulfil their function. In this respect, recent data has shown that IL-4 is not sufficient for CD4+ T helper 2 differentiation rather there is an additional requirement for IL-2 (592). This might reflect that only IL-2 can maintain sufficiently high rates of cell growth in CD4 T cells and that the role of IL-4 is to regulate differentiation of the cells. We performed a pilot experiment to probe this possibility. Thus OT-1 CD4+ splenic T cells were stimulated with cognate ova peptide and maintained in culture in the presence of 20ng/mL IL-2 ($CD4^{IL-2}$) or IL-4 ($CD4^{IL-15}$). Both cultures proliferated equally, but, $CD4^{IL-2}$ T cells were larger than $CD4^{IL-4}$ when analysed for FSC/SSC on the flow cytometer (data not shown). We would thus predict that IL-2 is the superior growth factor for antigen activated CD4+ T cells. The basis for this differential effect of IL-2 versus IL-4 for T cell growth needs further analysis but may reflect quantitative and kinetic differences in signal transduction as shown herein for IL-2 versus IL-15.

In summary we have compared a number of different γc cytokines for their ability to regulate T cell growth. Our studies indicate that IL-2 may be unique. This comparison of different γc cytokines was limited to studies of CD8+ T cells. Clearly it will be important to examine the role of these cytokines as growth regulators in other T cell populations including Th1/Th2 cells, regulatory T cells and NK/NKT cells. As well, these studies could be extended to other γc cytokines such as IL-21, which has been shown recently to synergise with IL-15 for CD8+ T cell activation (194). It would also be valuable to comprehensively analyse and compare the effects of different γc cytokines on amino acid transporter expression, amino acid uptake and incorporation into protein, glucose receptor expression, glucose uptake and importantly $PI(3,4,5)P_3$ levels in these different lymphocyte populations.

Final Discussion

The data in this thesis highlights differences between γc cytokines, specifically IL-2 and IL-15, in their role as T cell growth factors. The comparison of IL-2 and IL-15 came about because of data presented by Von Andrian's lab showing that either cytokine drove terminal differentiation of phenotypically divergent cell types, CD8+ effector (T_{EM}) and central (T_{CM}) memory T cells respectively (48, 51). This data was particularly interesting as IL-2 and IL-15 share two common receptor subunits (IL-2Rβ, IL-2Rγc) and have been shown to drive very similar transcriptional programs (205, 206). Thus the aim of the thesis was to conduct a kinetic analysis of IL-2 and IL-15 signalling in peptide activated CD8+ T cells to define divergence in signalling that could possibly be driving differentiation. The present data shows that IL-2 and IL-15 are equivalent mitogens for antigen stimulated CD8+ T cells but they are not equivalent growth factors and are strikingly distinct in their ability to control protein synthesis and T cell size. IL-2 and IL-15 were shown to induce protein synthesis in antigen activated T cells although protein synthesis driven by IL-2 is approximately two fold greater than that induced by IL-15. The ability of IL-2 to induce cell growth was thought to be associated with its role as a mitogen. However the level of cell growth induced by IL-2 seems to exceed that required for mitosis, as judged by the ability of IL-15 to sustain mitosis and T cell clonal expansion without maintaining T cells at the same size as IL-2. Hence the role of IL-2 as an effector CD8+ T cell growth factor may be relevant to its ability to sustain synthesis and production of effector molecules. The differential effect of IL-2 and IL-15 on general protein synthesis may help resolve the puzzle of how IL-2 and IL-15 differentially direct

CD8+ T cell fate (48, 51, 229), even though they initially activate common signal transduction pathways and induce very similar patterns of gene transcription (205, 206).

These data may help to understand the full power of IL-2 on T cell protein synthesis and may help explain why IL-2 can sustain T cell expansion *in vivo* even though it is not essential to initiate T cell cycle progression (129, 529). Also, this data may give some insight as to why IL-2 has a unique role *in vivo* as a regulator of peripheral immune homeostasis (132). In particular, it may help to explain why antigen stimulated T cells fail to secrete effector cytokines in the absence of IL-2 function even though they appear to undergo normal cell divisions (133).

In the present study we also compared the IL-2 growth responses of T cells to other members of the γc cytokine family, namely IL-4 and IL-7. It was clear that IL-2 was more potent than IL-4 and IL-7. The differential effects of γc cytokines on T cell size correlated with differences in their ability to maintain surface levels of the transferrin receptor CD71 and the amino acid transporter subunit CD98. Hence γc cytokines have different potentials for regulating protein synthesis and cell growth.

The present data show that the ability of T cells to maintain protein synthesis is driven by constant signal input and constant activation of PI3K, as removal of cytokine or inhibition of PI3K almost immediately abrogates amino acid uptake and amino acid incorporation into proteins. In particular the differential effects on T cell growth by IL-2 and IL-15 seems to correlate with differential kinetics of

PI3K signalling stimulated by these two cytokines. Thus IL-2 has the capacity to sustain PI3K signalling at high levels for several days whereas IL-15 only transiently induces high levels of PI3K signalling and very low levels during a sustained response. The major difference between IL-2 signalling is thus temporal and quantitative: IL-15 signalling is relatively transient whereas IL-2 signalling is sustained. These data reveal the importance of understanding the kinetics of signalling responses induced by different cytokines. Moreover, as the different cytokines exert their biological functions over several days it is necessary to examine signalling over long time courses rather than just focusing on immediate events.

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