Differential regulation of T cell growth by Interleukin 2 and IL-15

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Abstract

Interleukin 2 (IL-2) and IL-15 signal via the common gamma chain (γc) and IL-2 receptor β chain (CD122) subunits but direct distinct physiological and immunotherapeutic responses in T cells. The present study provides some insights about why IL-2 and IL-15 differentially regulate T cell function by revealing that these cytokines are strikingly distinct in their ability to control protein synthesis and T cell mass. IL-2 and IL-15 are shown to be equivalent mitogens for antigen stimulated CD8+ T cells but not equivalent growth factors. Antigen primed T cells cannot autonomously maintain amino acid incorporation or de novo protein synthesis without exogenous cytokine stimulation. Both IL-2 and IL-15 induce amino acid uptake and protein synthesis in antigen activated T cells however, the IL-2 response is strikingly more potent than the IL-15 response. The differential action of IL-2 and IL-15 on amino acid uptake and protein synthesis is explained by temporal
differences in signalling induced by these two cytokines. Hence the present results show that cytokines that are equivalent mitogens can have different potency in terms of regulating protein synthesis and cell growth.

**Introduction**

Interleukin 2 (IL-2) and IL-15 are critical but functionally distinct regulators of peripheral T lymphocyte proliferation and differentiation. IL-2 and IL-15 interact with T cells via cytokine receptor complexes consisting of the common gamma chain (γc) a common β chain subunit (CD122) and a unique α chain subunit that confers high affinity cytokine binding \(^1\,^2\). CD25 is the IL-2 receptor α chain, and IL-15 binds with high affinity to the unique IL-15 receptor α subunit (IL-15Rα) \(^3\,^4\). IL-2 was the first cytokine shown to drive mitosis in activated T cells \(^5\) but its mitogenic role can be supplanted by other γc cytokines *in vivo* \(^6\). Nevertheless, IL-2 has essential non redundant functions *in vivo* including the efficient production of effector cytotoxic T cells, the regulation of regulatory T cell survival \(^7\), maintenance of tolerance and sustaining T cell expansion \(^8\,^9\,^10\,^11\,^12\). IL-15 also has unique functions *in vivo* where it has been shown to be crucial for NK cell development and vital for the generation and maintenance of CD8+ memory T cells \(^1,^14\,^15\,^16\). The different physiological functions of IL-2 and IL-15 are reflected by their differential actions as immunotherapeutic agents \(^17\,^18\,^19\,^20\). In particular, IL-15 has been shown to act as a superior stimulus compared to IL-2 for the generation of memory and effector T cells following vaccination or adoptive transfer of tumour specific T cells \(^21\). Hence there is an increasing interest in the use of exogenous IL-15 rather than IL-2 for immunotherapy.

The different physiological and immunotherapeutic functions of IL-2 and IL-15 are not easy to explain in molecular terms. In fibroblasts and dendritic cells there is evidence that IL-15 receptor alpha subunits may signal to cell survival factors via pathways not used by IL-2 \(^22\,^23\). However, the immediate biochemical consequences of triggering T cells with IL-2 and IL-15 results in a common and indistinguishable biochemical response, a reflection of signalling through common γc/CD122 complex \(^24\,^25\). For example, IL-2 and IL-15 both
activate the Janus kinases Jak-1 and Jak-3 and the transcription factor STAT5. Moreover, cDNA microarray analysis has revealed that immediate T cell responses to IL-2 and IL-15 induce similar gene expression profiles. The commonality of immediate biochemical and transcriptional responses to IL-2 and IL-15 in T cells should mean that these cytokines dictate a common biological outcome. However, the fact they do not means there are differences in the signaling pathways triggered by IL-2 and IL-15 explaining their unique functions. In this context, one study has documented differential sensitivity of IL-2 and IL-15 induced T cell proliferation to the immunosuppressive agent rapamycin. This drug works by regulating the activity of the mammalian target of rapamycin (mTOR), a key regulator of mRNA translation and protein synthesis.

Historically, IL-2 was called T cell growth factor because of its ability to simultaneously induce cell cycle progression and induce an increase in cell size. However, activated CD8+ T cells maintained in the presence of IL-15 are described as small in comparison to IL-2 maintained cells. This supports the idea that IL-2 and IL-15 may differ in their ability to regulate T cell growth. In this context, analysis of IL-2 and IL-15 signal transduction has focused on their immediate biochemical and transcriptional programs and not on their effects on cell metabolism and 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, cellular energy and metabolism will influence how this genetic program is executed. Indeed, the term mitogen and growth factor are frequently used interchangeably to describe γC cytokines even though they are not synonyms. 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. Thus a potent mitogen may not be a potent growth factor. Accordingly, in the present study the actions of IL-2 and IL-15 on protein synthesis and cell growth of antigen activated T cells were compared. The results found that IL-2 and IL-15 are strikingly different growth factors for antigen activated T cells.
reflecting differences in the ability of these two cytokines to regulate cellular amino acid uptake and protein synthesis. The differential regulation of protein synthesis and T cell mass by IL-2 and IL-15 offers insights that could be a key to understanding the unique non-redundant actions of these cytokines.

Materials and methods

Mice

P14 TCR transgenic Mice were bred and maintained in the Cancer Research UK Biological Resources Unit and the Wellcome Trust Biocentre, University of Dundee in compliance with UK Home Office Animals (Scientific Procedures) Act 1986 guidelines. The P14 TCR recognises LCMV GP (33–41) (KAVYNFATM) in the context of H-2Db.

Cell culture

Spleens and lymph nodes were removed from 2-6 month old P14 LCMV TCR transgenic mice, mashed in cell strainers and suspended in RPMI with 10% FCS and 1μM GP (33-41) LCMV TCR specific peptide for 2 days. The LCMV GP (33–41) peptide, KAVYNFATM, was synthesised and purified in the Cancer Research UK Protein Production Laboratory. Thereafter, cells were washed out of peptide and cultured in recombinant human (rh) IL-2 (Proleukin, Chiron.B.V, Netherlands), (rh) IL-15 (Peprotech) or left in RPMI 1640/10% FCS (heat inactivated)/50μM βME as a control. Unless indicated differently, cytokine concentrations were 20ng/mL and cells were cultured at a density of 0.25-1 x 10^6/mL. To inhibit PI3K the inhibitor LY294002 (Promega) was added to cell cultures at a final concentration of 10μM.

Flow cytometric analysis

Rat anti-mouse antibodies (Pharmingen, San Diego, CA) were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC). TriColor (tricol) was obtained from Caltag. Anti-mouse IL-15Rα antibody from R&D systems, phospho-S6 ribosomal protein (Ser235/236)
antibody from cell signalling technology and secondary anti-goat and anti-rabbit FITC conjugated antibodies were from Jackson ImmunoResearch labs. Samples were stained for surface expression of the following markers with antibody clonal typing in parentheses: CD25 (IL-2Rα chain, p55, 7D4, 3C7), CD62L (L-Selectin, LECAM-1, Ly-22, MEL-14), common γ chain (γc) (4G3), IL-15Rα (R&D systems), CD71 (transferrin receptor, C2F2), CD98 (42F, H202-141). For identification of naïve P14 TCR transgenic CD8+ T cells in spleen and lymph node and P14 TCR transgenic mouse blood typing, the following markers were used: Thy1.2 (CD90.2, 53-2.1), CD8a (Ly-2, 53-6.7, tricol 5H10), CD4 (L3T4, tricol RM4-5, GK1.5), Vα2 (T Cell Receptor, B20.1), Vβ8 (T Cell Receptor, F23.1). In most cases cells were blocked for 30 min at 4 °C with mouse Fc Block (CD16/32, Fc γ III/II Receptor, 2.4G2). Cells were then stained with saturating concentrations of antibody at 4 °C for 20-60 min at 1 x 10⁶ cells per sample and washed before analysis 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.

**Phospho-S6 ribosomal protein intracellular staining**

T cells were washed and fixed in 0.5% PFA for 15 minutes at 37°C, washed twice in PBS, and permeabilised for 15 minutes in 90% methanol at -20°C. Cells were washed twice in PBS and blocked for 10 minutes at room temperature (RT) in BSA buffer (0.5% bovine serum albumin in PBS). Cells were incubated with 1:100 dilution of primary anti-phospho-S6 (Cell Signalling Technologies) in BSA buffer for 30 minutes at RT. Cells were washed once with BSA buffer and incubated with 1μg of FITC conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) for 30 minutes at RT in the dark. Samples were washed in BSA buffer and analysed on a FACS Calibur. Pharmacological stimulation for 30 minutes 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.
Proliferation assays

Antigen activated P14 LCMV CD8+ T cells were re-suspended in RPMI1640/10% FCS/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 IL-2, IL-15 or medium alone. Cells were then cultured at 37°C in a 5% CO₂ humidified incubator for the indicated time. Prior to harvesting 1µCi of tritiated (³H) thymidine (Amersham, UK) was added to each well and cells were 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.

Amino acid uptake into cells

The rate of amino acid uptake into antigen stimulated P14 LCMV CD8+ T cells in response to different concentrations of IL-2, IL-15 or medium alone was assayed by re-suspending antigen activated CD8+ T cells in RPMI 1640/10% FCS/50µM βME, and seeding in triplicate at 5 x 10^4-1 x 10^6 cells/well in 96 well-flat bottom microtitre plates. Cells were then cultured at 37°C in a 5% CO₂ humidified incubator for the indicated time. Tritiated (³H) 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.

Amino acid incorporation into cellular protein

To determine amino acid incorporation into cellular protein, antigen stimulated P14 LCMV CD8+ T cells were cultured in 20ng/mL IL-2, IL-15 or medium alone at 10^6 cells/mL for indicated time points. Cells were pulsed for 12 hours, prior to harvest, with 5µCi/mL tritiated (³H) amino acid mix (Amersham, UK). Cell pellets were harvested in triplicate and lysed in 1mL of buffer (final 10mL solution: 50mM HEPES at pH 7.4, 150mM NaCl, 10mM NaF, 10mM iodoacetamide (IAA), 1mM phenyl methyl sulphonyl fluoride (PMSF), 1 mM
sodium orthovanadate, 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) for 20 min at 4 °C. The resulting extract was centrifuged at 15,000g for 15 min at 4 °C, protein precipitated in equal volume of 70% acetone and incorporated radioactivity quantified on a β scintillation counter.

**Protein Content**

Cell pellets containing $10^6$ cells was lysed at 4 °C in 1mL of buffer (final 10mL solution: 50mM HEPES at pH 7.4, 150mM NaCl, 10mM NaF, 10mM iodoacetamide (IAA), 1mM phenyl methyl sulphonyl fluoride (PMSF), 1 mM sodium orthovanadate, 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). Protein content quantified using the BIORad protein assay dye reagent, referenced against a standard curve of Bovine Serum Albumin.

**Results**

**IL-2 and IL-15 are potent T cell mitogens but differentially effect T growth in the context of T cell size and morphology.**

Experiments to compare the ability of IL-2 and IL-15 to regulate growth or cell mass used antigen activated CD8+ T cells from the P14 TCR transgenic mouse model where peripheral CD8+ T cells express a $\alpha_2/\beta_8.1$ TCR specific for the lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) peptide, gp33-41, presented by the MHC class I molecule, H-2Db. The rationale for this choice of model is that IL-2 and IL-15 are equivalent mitogens for antigen primed P14 CD8+ T cells in vitro but differentially regulate their differentiation. Antigen primed P14 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. Splenocytes were isolated from P14 LCMV mice and cultured with gp33-41 (1uM) peptide for
two days to generate activated CD8+ T lymphoblasts. Figure 1A shows both IL-2 and IL-15 induced equivalent DNA synthesis in antigen primed CD8+ T cells and could sustain exponential division of these cells over 5-6 days. (Figure 1B). During the early phase of this response the cell doubling time of T cells maintained in IL-2 or IL-15 was equivalent and resulted in a 200-500 fold increase in cell numbers. Thereafter, at day 6-8, responses to the two cytokines diverge: T cells maintained in IL-2 stop proliferating and decline in number. This decline in cell numbers is due to increased apoptosis 39. In contrast, T cells maintained for a similar time in IL-15 slow in their division rate but survive for prolonged periods (Figure 1B).

During the exponential phase of cell proliferation cells cultured in IL-2 or IL-15 had a visually distinct size and morphology (Figure 2). Antigen primed T cells proliferating in response to IL-2 are large granular blasts whereas T cells proliferating in IL-15 are smaller and less granular. These differences are easily visible by light microscopy (Figure 2A) and by flow cytometric analysis of forward light scatter (FSC) and side light scatter profiles (SSC) of IL-2 and IL-15 cultured T cells (Figure 2B). Flow cytometric analysis of forward and side scatter profiles estimates 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 data in Figure 2C show the protein content of T cells maintained in IL-2 or IL-15. T cells cultured with IL-2 have a substantially higher protein content than cells cultured in IL-15.

IL-2 and IL-15 differentially control cell size of antigen activated CD8+ T cells.

Antigen activation of naïve T cells causes the cells to increase in size. The dramatic size difference of antigen activated versus primary naive T cells is readily demonstrated by flow cytometric analysis of forward and side light scatter (FSC, SSC) profiles, shown by the comparison of ex vivo CD8+ naïve P14 TCR transgenic T cells and T cells stimulated for two days by the LCMV gp33-41 peptide complexed antigen presenting cells (Figure 3A & 3B). The size difference between quiescent CD8+ T cells and activated T cell blasts is
further defined by the 2-4-fold increase in cellular protein levels after resting CD8+ T cells are activated (Figure 3C).

The size and morphology of antigen activated T lymphoblasts is comparable to effector T cells cultured in IL-2 whereas T cells cultured long term with IL-15 are markedly smaller (Figure 2). Hence IL-2 can maintain the size of antigen activated T cells whereas IL-15 cannot. The data in Figure 3D show the kinetics with which antigen primed T cells cultured in IL-15 reduce in size over 24-72 hours. 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 (Figure 3D). These changes are seen within 24–48 hours of antigen depravation whereat later time points the cells die. When antigen activated T cells are cultured with IL-2 they maintain cell size (Figure 3D). In contrast, antigen activated T cells cultured in IL-15 decrease in size and protein content. This is not an immediate response as with complete withdrawal of cytokine but occurs over a period of 48-72 hours (Figure 3B). The first indication that IL-15 cannot maintain the morphology of antigen activated T cells can be judged by comparing the side scatter profiles of T cells maintained in IL-2 or IL-15 for 24 hours; at this point the cells have a 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 a lower protein content than T cells maintained for an equivalent time in IL-2 (Figure 3D). 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 (Figure 3E).

**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 uptake 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 Figure 4A show that an antigen activated T cell cannot autonomously maintain amino acid uptake when cultured in medium alone. 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 with IL-15 (Figure 4A). 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 (3H) amino acids into cellular proteins was compared, the IL-2 response markedly exceeded the IL-15 response (Figure 4B). Differences between IL-2 and IL-15 in their ability to regulate cell size 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 (Figure 1A) 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) (Figure 4C).

**IL-2 and IL-15 differentially regulate expression of transferrin receptor**

**CD71 and amino acid transporter subunit CD98.**

Amino acid uptake in haematopoietic cells is regulated by amino acid transporter complexes that include CD98 (42F) as a key component. We therefore assessed if differences between IL-2 and IL-15 in terms of their ability to regulate amino acid uptake was explained by differences in their ability to regulate expression of CD98. As well, 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 crucial metabolic reactions, to the cell interior. Accordingly we compared the effect of IL-2 and IL-15 on expression of CD71 the transferrin receptor. The data in Figure 5A show that antigen activated CD8+ T cells maintained in IL-2 have high levels of surface CD98 compared to cells cultured with no cytokine (medium control). Similarly, 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 (Figure 5B). The data show that levels of CD98 and transferrin receptors in T cells maintained in IL-15 are reduced compared to T cells cultured with IL-2 (Figure 5A & 5B). The level of transferrin receptor expression in a T cell cultured with IL-15 for 24 or 48 hours is also lower than in T cells cultured in the presence of IL-2. T cells cultured in IL-15 do not globally down regulate all surface receptors: levels of L-selectin (CD62L) and common γ chain cytokine receptor subunit are increasing over 24-48 hours in the presence of IL-15 or medium alone when compared to CD8+ T cells grown in the presence of IL-2 (Figure 5C).

The role of PI3K in IL-2 regulation of protein synthesis and T cell growth

The expression of CD98 in the IL-3 responsive haematopoietic pro-B cell line FL5.12 is controlled by the serine kinase AKT or Protein Kinase B (PKB) which is activated by Phosphatidylinositol(3,4,5)triphosphate (PtdIns(3,4,5)P₃) the lipid product of phosphoinositide 3-kinases (PI3Ks). Moreover, an evolutionarily conserved pathway for the regulation of cell metabolism and growth by PI3K is mediated by the serine/threonine kinase Phospholipid Dependent Kinase 1 (PDK1) which regulates the activity of PKB/Akt and the 70-kilodalton ribosomal S6 kinase 1 (S6K1). The importance of PDK1 for the regulation of T cell size has been explored in transgenic mouse models. Accordingly, T cell specific deletion of PDK1 has an immediate impact on thymocyte growth and results in a cell size reduction in proliferating pre-T cells. Conversely, expression of a constitutively active membrane targeted PKB in T cells of transgenic mice causes an increase in T cell size. We therefore considered the possibility that differences between IL-2 and IL-15 in terms of their ability to regulate CD98 expression and amino acid uptake and protein synthesis reflected differences between these two cytokines in terms of their ability to induce PI3K/PDK1/PKB signalling. To test this hypothesis we first assessed whether CD98 expression on T cells is regulated by PI3K. Figure 6A shows CD98 levels are markedly reduced when IL-2 cultured antigen activated CD8+ T cells are treated with a pharmacological inhibitor of PI3K LY294002 (LY). CD71 expression is also reduced in IL-2
activated T cells cultured with LY294002 (Figure 6B). The impact of
LY294002 on the ability of IL-2 to sustain T cell growth and cell size was also
examined. The data in Figure 6C show the forward light scatter (FSC) profile
of antigen activated T cells maintained in IL-2 in the presence or absence of
LY294002. IL-2 activated T cells treated with LY294002 are smaller than cells
exposed to IL-2 alone. The data (Figure 6D & 6E) also show that LY294002
inhibited IL-2 induced amino acid uptake and incorporation into cellular
protein. The protein content of IL-2 activated cells is reduced approximately 2-3
two fold by LY294002 treatment although it is not reduced to the lower level of
protein present in T cells cultured with no cytokine (Figure 6F). In further
experiments the impact of LY294002 on IL-15 induced protein synthesis was
examined. The data show that LY294002 inhibited IL-15 induced cellular
amino acid uptake (Figure 6G) and correspondingly reduced cell size (data
not shown). IL-2 and IL-15 are potent survival factors for antigen primed
CD8+ T cells and in many cells PI3K signals are crucial regulators of survival
responses. IL-2 or IL-15 cultured T cells do not die in the presence of
LY294002 but persist as small rounded cells (Figure 6C). LY294002 thus
blocks T cell growth but not survival. Moreover the effects of LY294002 are
reversible: T cells cultured with IL-2 plus LY294002 for two days can regain
cell size and proliferate if washed free of drug and re-cultured with IL-2 (data
not shown).

Differential kinetics of IL-2 and IL-15 induced Phospholipid Dependent
Kinase 1 (PDK1) signalling.

The results in Figure 6 show that T cell size, PI3K sensitive pathways in T
cells regulate amino acid transporters and uptake.
Hence differences between IL-2 and IL-15 in terms of their ability to regulate
cell growth could reflect that IL-2 and IL-15 differ in their ability to induce or
sustain PI3K signalling. The downstream effector of PI3K in terms of cell
growth regulation is the PDK1/PKB pathway and a sensitive and quantitative
method to monitor this pathway is to analyse phosphorylation of the ribosomal
S6 subunit by flow cytometric analysis of intracellular staining with specific
phospho-S6 antisera. S6 phosphorylation is mediated by S6K1, which
needs phosphorylation by PDK1 to be functional. S6 phosphorylation is also regulated by another PDK1 substrate PKB via TSC-1/2 mediated regulation of S6K1 function. Flow cytometric analysis phospho-S6 thus looks in single cells at phosphorylation of a downstream target of PI3K/PDK1/PKB/S6K1 pathways at a point where there has been considerable signal amplification.

The results in Figure 7A show that ex vivo naïve CD8+ T cells dramatically increase levels of phospho-S6 after stimulation with LCMV gp33-41 peptide for 2 days. These high levels of phospho-S6 are lost if cells are removed from antigen and cultured in medium alone, indicated in the top panel of Figure 7B. Phospho-S6 levels are maintained when antigen activated T cells are cultured with IL-2 (Figure 7B middle panel) and this IL2 response is dependent on activation of PI3K as co-culture of T cells with IL-2 and the PI3K inhibitor LY294002 abrogates phospho-S6 staining (Figure 7C). Comparison of IL-2 and IL-15 regulation of the phospho-S6 pathway showed an equal ability to sustain S6 phosphorylation at an early 6hour time point (Figure 7B, left hand panel). However, during prolonged exposure to cytokine only IL-2 maintained high levels of phospho-S6 whereas the IL-15 response was more transient (Figure 7B). Thus T cells maintained in IL-15 for 12-24 hours have markedly lower levels of phospho-S6 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 alone but considerably lower than cells cultured in IL-2 (Figure 7B).

We considered the possibility that the difference between IL-2 and IL-15 in terms of their ability to regulate phosphorylation of S6 protein reflected that CD8+ T cells maintained in IL-15 had lower levels of the substrate S6 protein than cells grown in IL-2. To explore this possibility we examined the ability of T cells maintained in IL-2 or IL-15 to induce S6 phosphorylation in response to pharmacological stimulation with the phorbol ester, phorbol 12.13 dibutyrate (PdBu). The data in Figure 7D show that the levels of phosphoS6 in PdBu treated cells maintained in IL-2 or IL-15 are comparable. Hence T cells maintained in IL-15 or IL-2 have similar levels of S6 phosphorylation when activated pharmacologically and thus similar levels of total S6 protein.
**Regulation of IL-2 and IL-15 receptors**

Differences in the kinetics of IL-2 and IL-15 induction of phosphoS6 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.\(^{5,49,50}\) The high affinity binding of IL-2 is mediated by a complex of; the common cytokine receptor \(\gamma\) chain (\(\gamma_c\)), CD122 (the IL-2 receptor \(\beta\) chain) and CD25 (the unique IL-2 receptor \(\alpha\) chain). IL-15 interacts with cells via binding to the unique IL-15 receptor \(\alpha\) subunit (IL-15R\(\alpha\)), which presents IL-15 in trans to \(\gamma_c\) and \(\beta\) (CD122) subunits present on nearby cells.\(^{3,4}\) IL-2 binding to its receptor triggers receptor internalisation and ligand degradation but simultaneously up regulates expression of CD25, 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.\(^{52}\)

To examine the possibility that IL-15 down regulates expression of the IL-15 receptor \(\alpha\) 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. Surface levels of CD25, the unique IL-2 receptor \(\alpha\) chain, are markedly up regulated when antigen activated CD8+ T cells are grown in IL-2 versus media alone (Figure 8A). IL-15 is not as potent as IL-2 at maintaining IL-2 receptor levels but still stimulates CD25 expression when compared to media alone. Activated CD8+ T cells also express IL-15R\(\alpha\) but surface levels of this receptor are not maintained in the presence of IL-15 (Figure 8B). IL-15R\(\alpha\) 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 \(\alpha\) 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.

Discussion

IL-2 and IL-15 are potent mitogens for antigen stimulated CD8+ T cells but differentially control T cell differentiation. These two cytokines also have a differential impact when used therapeutically to modify immune responses. The present study provides some new insights into why IL-2 and IL-15 differentially regulate T cell function by revealing that these cytokines are strikingly distinct in their ability to control protein synthesis and T cell size. IL-2 and IL-15 are thus equivalent mitogens for antigen stimulated CD8+ T cells but not equivalent growth factors. Antigen activated CD8+ T cells are large blastoid cells but 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 amino acid transporter subunit CD98. IL-2 can induce high levels of CD98 expression and amino acid uptake compared to the relatively weak effects of IL-15. 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.

IL-15 can sustain mitosis and T cell clonal expansion without maintaining T cells at the same size as IL-2. Hence, the level of cell growth induced by IL-2 exceeds that required for mitosis and 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, even though they initially activate common signal transduction pathways and induce very similar patterns of gene transcription. 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 its 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. 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. 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 even though they appear to undergo normal cell divisions.

The present data show that the ability of T cells to maintain protein synthesis is driven by constant signal input, as removal of cytokine immediately abrogates amino acid uptake and amino acid incorporation into proteins. The differential actions of IL-2 and IL-15 on amino acid uptake and T cell growth correlates with the differential kinetics of the PI3K/PDK1 signalling pathway stimulated by these two cytokines. Hence, in antigen activated T cells both IL-2 and IL-15 stimulate PI3K/PDK1 mediated signal transduction pathways that culminate in phosphorylation of the ribosomal S6 subunit. 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 PI3K signalling at high levels whereas IL-15 only transiently induces high levels of PI3K signalling and during a sustained response gives a low level of S6 phosphorylation. The major difference
between IL-2 and IL-15 signalling to PKB and S6K1 is thus temporal and quantitative.

Why is IL-15 activation of the S6 pathway transient whereas the IL-2 response is sustained? We would exclude that IL-15 up regulates a negative feedback pathway that globally antagonizes PI3K signals because T cells maintained in IL-15 can immediately up regulate S6 phosphorylation and increase cell size if re-exposed to IL-2. It is possible 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. 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. However, an explanation for the relative transience of the IL-15/S6 response compared to the IL-2 sustained response also resides in differences in the way these two cytokines can modulate expression of their own receptors. 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 have been shown to 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 IL-7 signaling, 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.

IL-2 and IL-15 induce different biological functions in T cells where IL-2 promotes differentiation of effector CTL whereas IL-15 promotes memory T cell development. Also, IL-15 is increasingly considered to be superior to IL-2 for immunotherapy in the context of dose toxicity. Hence, are the different
biological actions of IL-2 and IL-15 explained by temporally quantitative
differences in signal transduction by these cytokines? Data that support this
theory includes the fact that low doses of IL-2 mimic IL-15 and induce the
differentiation of memory T cells rather than effector CTL \(^{29}\). It is also known
that low doses of IL-2 are more valuable immunotherapeutically than high
doses \(^{54}\). The relevance of these data to the present study is that low doses of
IL-2 result in transient signalling because activated T cells consume IL-2 (i.e.
internalise and degrade) and at low doses rapidly deplete the cytokine from
culture medium and hence make it only transiently available to the T cell
population.

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 differences between IL-2 and
IL-15 are explained by temporal differences in signalling that translates into
differences in protein synthesis. In particular, the ability of IL-2 to sustain high
levels of PI3K/PDK1 signal transduction allows this cytokine to sustain T cell
protein synthesis at high levels for a prolonged period. The differential effects
of IL-2 and IL-15 on T cell protein synthesis can promote rational decisions in
choosing IL-2 versus IL-15 for immunotherapy. IL-2 will be of use for short-
term expansion of potent effector T cells but less valuable for immune
replenishment because it promotes a level of protein synthesis in excess of
that needed for T cell mitosis. This is beneficial in the short term but very
energetically demanding which probably explains why T cells cultured in IL-2
are very susceptible to apoptosis. IL-15 appears to more closely match levels
of protein synthesis to mitosis and would hence be the cytokine of choice for
efficient proliferative expansion of T cells.

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Figure legends

Figure 1: IL-2 and IL-15 are equivalent mitogens for antigen primed CD8+ T cells

A) Data show tritiated (³H) thymidine incorporation into P14 LCMV CD8+ T cells primed with LCMV gp33-41 peptide for two days and thereafter cultured in the indicated concentration of IL-2 (CD8IL-2), IL-15 (CD8IL-15) or in medium alone for 24 hours. B) Cell numbers of antigen activated CD8+ T cells maintained in exponential proliferation with 20ng/mL IL-2 (CD8IL-2), 20ng/mL IL-15 (CD8IL-15) and medium over the indicated period of days. Graphs represent 7 or more experiments.

Figure 2: Antigen primed T cells cultured in IL-2 are bigger than T cells cultured in IL-15

A) Live low light digital camera images of 2-day antigen primed CD8+ T cells cultured for 5 days in either 20ng/mL IL-2 (CD8IL-2) or 20ng/mL IL-15 (CD8IL-15). Cells were allowed to settle on glass cover slips coated with 5ng/mL of recombinant mouse ICAM-1/Fc chimera (R&D Systems), B) FACS dot plots of
forward light scatter (FSC) and side light scatter (SSC) profiles and C) Cellular protein content. Dot plots and graph are representative of 5 or more experiments. P values were lower than 0.05 for protein concentrations.

Figure 3: Kinetics of the change in cell size of antigen activated CD8+ T cell cultures stimulated with IL-2 or IL-15

A, B) Data show dot plots and histograms of forward light scatter (FSC) and side light scatter (SSC) of naïve P14 LCMV transgenic CD8+ T cells or P14 LCMV CD8+ T cells activated with LCMV gp33-41 peptide for 2 days. Cells represented in histogram profiles were gated on size, as indicated in dot plots, and equal expression levels of the CD8a surface molecule (data not shown). C) Cellular protein content (mg/mL) of $10^6$ naïve P14 LCMV transgenic CD8+ T cells or P14 LCMV CD8+ T cells activated with LCMV gp33-41 peptide for 2 days. D) Data show FSC, SSC and cellular protein content of $10^6$ peptide 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). E) FACS dot plots with FSC, SSC profiles of antigen activated P14 LCMV CD8+ T cells cultured in 20ng/mL IL-15 (CD8 IL-15) for 3 days. After 3 days in culture CD8 IL-15 cells were washed 3-4 times (to remove exogenous cytokine) and re-cultured in 20ng/mL IL-2 (CD8 IL-15 + IL-2) or again in 20ng/mL IL-15 (CD8 IL-15) for a further 48 hours. As a comparison FSC/SSC profiles of antigen activated P14 LCMV CD8+ T cells maintained only in IL-2 (CD8 IL-2) for 5 days is shown. All histograms, dot plots and graphs are representative of 4 or more experiments. P values were equal to or below 0.05 for concentration differences equal to or greater than 2 fold.

Figure 4: IL-2 and IL-15 regulation of amino acid (aa) uptake into cells and incorporation into cellular proteins

A) Tritiated ($^3$H) amino acid uptake by P14 LCMV CD8+ T cells activated with LCMV gp33-41 peptide for 2 days and thereafter cultured for 24 hours with the indicated concentrations of IL-2 or IL-15. B) Incorporation of ($^3$H) tritiated amino acid into precipitated cellular protein of antigen activated P14 LCMV CD8+ T cells cultured for 24 hours 20ng/mL IL-2, 20ng/mL IL-15 or medium alone. C) Cellular DNA content of antigen activated P14 LCMV CD8+ T cells
cultured for 24 hours with medium, 20ng/mL IL-2 or 20ng/mL IL-15. Graphs are representative of 4 or more experiments and p values were estimated to be below 0.05.

**Figure 5: Expression levels of amino acid transporter CD98 (42F) and transferrin receptor CD71.**
A), B) FACS histograms of the expression of CD98 and CD71 on 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 24-48hrs. C) FACS histograms show expression levels of L-Selectin (CD62L) and the common γc chain cytokine receptor subunit on 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 24-48hrs. Histograms are representative of 7 or more experiments.

**Figure 6: The role of PI3K in regulating protein synthesis and cell size of activated T cells**
A), B) FACS histograms show expression of the amino acid transporter subunit CD98 and the transferrin receptor CD71 on the surface of antigen primed P14 LCMV CD8+ T cells cultured in medium alone, 20ng/mL IL-2 (CD8^{IL-2}) or 20ng/mL IL-2 (CD8^{IL-2}) plus 10μM LY294002 a PI3K inhibitor for 24 hours. C) FACS dot plots and histograms of forward light scatter and side light scatter (FSC/SSC) profiles of antigen primed P14 LCMV 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. D) Incorporation of tritiated (3H) amino acid (aa) into precipitated cellular protein of antigen primed P14 LCMV CD8+ T cells maintained for 48 hours in medium, 20ng/mL IL-2 or 20ng/mL IL-2 plus 10μM LY294002 a PI3K inhibitor. E) Percent maximum values of tritiated (3H) amino acid (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. F) Protein concentration of antigen primed P14 LCMV CD8+ T cells maintained for 48 hours in medium, 20ng/mL IL-2 (CD8^{IL-2}) or IL-2 plus 10μM LY294002. G) Percent maximum values of tritiated (3H) amino acid (aa) taken up by antigen primed P14 LCMV CD8+ T cells
maintained for 48 hours in medium or the indicated concentration of IL-15 plus or minus 10μM LY294002. Dot plots; histograms and graphs are representative of 5 or more experiments where p values were estimated around 0.001 for 6D, E, G and 0.05 for 6F.

Figure 7: IL-2 and IL-15 regulation of S6 ribosomal protein phosphorylation
Flow cytometric analysis of phospho-S6 ribosomal protein in: A) Naïve CD8+ T cells, 2 day antigen primed P14 LCMV CD8+T cells and B) antigen primed CD8+ T cells maintained for a further 6-24 hours in medium, 20ng/mL of IL-2 (CD8IL-2) or 20ng/ml of IL-15 (CD8IL-15), C) Antigen primed P14 LCMV CD8+ T cells maintained for 24 hours in medium or 20ng/mL IL-2 (CD8IL-2) plus or minus 10μM LY294002 a PI3K inhibitor, D) Antigen primed P14 LCMV CD8+ T cells maintained for 24 hours in 20ng/mL of IL-2 (CD8IL-2) or 20ng/mL of IL-15 (CD8IL-15) followed by no further treatment or activation with 20nM of PdBu for 30 minutes prior to analysis. FACS histograms are representative of 5 or more experiments.

Figure 8: Expression levels of the cytokine receptor unique α chains IL-2Rα (CD25) and IL-15Rα on T cells cultured in IL-2 or IL-15
A), B) FACS histograms show expression of IL-2Rα (CD25) and IL-15Rα subunits on antigen primed P14 LCMV CD8+T cells cultured in 20ng/mL IL-2 (CD8IL-2), IL-15 (CD8IL-15) or medium alone for 24 hours.
1A

$^{3}$H thymidine incorporation over 24 hrs (c.p.m x 10$^3$)

Cytokine (ng/mL)

1B

Cell number x 10$^6$

Days in culture
2A  
CD8 IL-2 T cells  
CD8 IL-15 T cells

2B  
SSC  
FSC

2C  
Protein (mg/10^6 cells)  
CD8 IL-2
CD8 IL-15

For personal use only.
3A

ex vivo CD8+ T cells

Peptide Stimulated T cells

3B

MFI:
- 275
- 420

MFI:
- 189
- 491

3C

Protein (mg/10⁶ cells)

- ex vivo CD8+ T cells
- Peptide activated T cells
Washout IL-15 add IL-2 for 48hrs

Continue to maintain in IL-15 for 48hrs

Maintained in IL-2 only

CD8 IL-15 + IL-2

CD8 IL-15

CD8 IL-2
4A

\[ \text{H}^3\text{H}-\text{aa uptake (c.p.m x 10}^5\text{ cells)} \]

\begin{center}
\begin{tabular}{c|c|c}
\text{Cytokine ng/mL} & CD8\text{IL-2} & CD8\text{IL-15} \\
\hline
0 & 500 & 500 \\
5 & 1000 & 1500 \\
10 & 1500 & 2000 \\
15 & 2000 & 2500 \\
20 & 2500 & 3000 \\
25 & 3000 & 3500 \\
\end{tabular}
\end{center}

4B

\[ \text{H}^3\text{H}-\text{aa incorporated into protein (c.p.m x 10}^6\text{ cells)} \]

\begin{center}
\begin{tabular}{c|c|c}
\text{Cytokine ng/mL} & Medium & CD8\text{IL-15} & CD8\text{IL-2} \\
\hline
0 & 0 & 0 & 0 \\
5 & 4000 & 12000 & 160000 \\
10 & 8000 & 16000 & 320000 \\
15 & 12000 & 24000 & 480000 \\
20 & 16000 & 32000 & 640000 \\
25 & 20000 & 40000 & 800000 \\
\end{tabular}
\end{center}

4C

\[ \text{CD8IL-2} \]

\begin{center}
\begin{tabular}{c|c|c}
\text{Relative cell count} & 0 & 20000 \\
\end{tabular}
\end{center}

\[ \text{CD8IL-15} \]

\begin{center}
\begin{tabular}{c|c|c}
\text{Relative cell count} & 0 & 20000 \\
\end{tabular}
\end{center}

\[ \text{Medium} \]

\begin{center}
\begin{tabular}{c|c|c}
\text{Relative cell count} & 0 & 20000 \\
\end{tabular}
\end{center}
5A  CD98 (42F)

MFI:
- Medium: 205
- CD8^{IL-15}: 354, 703
- CD8^{IL-2}: 489

24hrs 48hrs

5B  CD71 (Transferrin)

MFI:
- Medium: 352, 844, 1319
- CD8^{IL-15}: 48, 438, 1383

24hrs 48hrs

5C  CD62L (L-Selectin)

MFI:
- Medium: 30
- CD8^{IL-15}: 26, 19
- CD8^{IL-2}: 11

24hrs 48hrs

Common γ chain

MFI:
- Medium: 25, 28, 11
- CD8^{IL-15}: 31, 32, 9

24hrs 48hrs
7A

Ex vivo CD8+ T cell

Peptide activated T cells

Phospho-S6

7B

6hrs 12hrs 24hrs

MFI:141

Medium

MFI:242

CD8IL-2

MFI:158

MFI:127

CD8IL-15

MFI:183

MFI:67

Phospho-S6

7C

CD8IL-2 +10μM LY

Medium

Phospho-S6

7D

IL-2 (24hrs)

MFI:180

MFI:127

IL-15 (24hrs)

MFI:173

MFI:68

Phospho-S6
8A  **IL-2Rα (CD25)**  **IL-15Rα**

- **IL-2** for 24hrs
- **MFI:**
  - Medium: 66
  - CD8\(^{IL-2/IL-15}\) as indicated: 709

8B  **IL-2Rα (CD25)**  **IL-15Rα**

- **IL-15** for 24hrs
- **MFI:**
  - Medium: 49
  - CD8\(^{IL-2/IL-15}\) as indicated: 185
Differential regulation of T cell growth by Interleukin 2 and IL-15

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