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Signaling and Function of Interleukin 2 in T lymphocytes

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Abstract

The discovery of Interleukin 2 (IL-2) changed the molecular understanding of how the immune system is controlled. IL-2 is a pleiotropic cytokine, and dissecting the signaling pathways that allows IL-2 to control the differentiation and homeostasis of both pro- and anti-inflammatory T cells is fundamental to determining the molecular details of immune regulation. The IL-2 receptor couples to JAK tyrosine kinases and activates the STAT5 transcription factors. However, IL-2 does much more than control transcriptional programs; it is a key regulator of T cell metabolic programs. The development of global phosphoproteomic approaches has expanded the understanding of IL-2 signaling further, revealing the diversity of phosphoproteins that may be influenced by IL-2 in T cells. However, it is increasingly clear that within each T cell subset, IL-2 will signal within a framework of other signal transduction networks that together will shape the transcriptional and metabolic programs that determine T cell fate.

The history of IL-2

Interleukin-2 (IL-2), a 15.5 kDa variably glycosylated globular protein, was first described as T cell growth factor (TCGF), a potent mitogen and growth regulator of T cells in vitro (1-6). The discovery of IL-2 stemmed from work that documented that conditioned media from lymphocytes activated polyclonally with plant lectins, such as phytohaemagglutinin, contained a protein that could support the proliferative expansion of cytotoxic T lymphocytes and the isolation of antigen-specific T cell clones (1). In seminal work, the group led by Kendal Smith used rigorous protein enrichment protocols to purify TCGF (IL-2) to homogeneity and generate bioactive, biosynthetically-radiolabeled, TCGF (7, 8). The availability of radiolabeled TCGF/IL-2 made it possible to explore how this cytokine interacted with T cells. This led to a fundamental discovery: the biological effects of TCGF were mediated by a high affinity cytokine receptor that was expressed selectively on T cells activated via their T cell antigen receptor, thereby ensuring the immune specificity of IL-2 actions (5, 6).

During the 1980s, the generation of monoclonal antibodies against T cell populations accelerated many new discoveries; in 1982, an antibody called anti-Tac was proposed to be a candidate IL-2 receptor antibody because it could block the binding of IL-2 to T cells. In addition, the expression of the Tac antigen on immune activated T cells coincided with the expression of the high affinity IL-2 receptor (9). However, there were discrepancies to be accounted for, such as the observation that activated T cells expressed a maximum of $4-8 \times 10^3$ high affinity IL-2 binding sites, but much higher numbers of Tac antigen ($40-100 \times 10^3$) binding sites (9). The cloning of the Tac antigen and its expression in heterologous cells then revealed that the Tac antigen was able to bind IL-2, but with low affinity (10, 11). The search was then on for other components that could confer the high affinity binding of IL-2 to its receptor. After much work, it became clear that the high affinity IL-2 receptor (IL-2R) comprised of three

polypeptides: Tac antigen (now known as CD25 or the IL-2 receptor alpha chain, IL-2R α), an IL-2 receptor beta chain (CD122 or IL-2R β) and a gamma subunit (γ_c , or CD132) (12-15). The co-expression of all three subunits is needed to confer high affinity IL-2 binding to a cell. The discrepancy in Tac antigen numbers and high affinity IL-2 binding sites was explained by the limiting numbers of IL-2R β / γ_c subunits. Subsequently, the expression of IL-2 α has been quantified and shown to exceed the levels of IL-2R β and/or γ_c using flow cytometry (16) and mass spectrometry (17).

The discovery of the IL-2/IL-2R system promoted an explosion of work to purify and identify other cytokines and their receptors. This work has exposed a complex system in which the γ_c from the IL-2R functions as a common subunit for multiple cytokines including IL-7, IL-4, IL-9, IL-15 and IL-21; and where the γ_c subunit and the IL-2R β subunit, in complex with the unique IL-15R α -chain, form the receptor for IL-15 (18). The discovery and biochemical characterization of IL-2, and the experiments to define the IL-2 receptor, were instrumental to effecting a change in the way immunologists viewed immunoregulation: they highlighted the fundamental importance of secreted cytokines for the control of T cell proliferation and differentiation. Indeed, it was quickly recognized that the loss of the γ_c was the molecular basis for X-linked severe combined immunodeficiency disease (XSCID) (19).

The discovery of IL-2 has had a significant impact on immunology research. Experiments to probe the molecular basis for IL-2 gene transcription led to the discovery of key antigen receptor signal transduction pathways, including the calcium/calcineurin pathway that controls the nuclear translocation of the transcription factor nuclear factor of activated T cells (NFAT) (20). Additionally, the importance of IL-2 as an immunomodulatory cytokine, and as a valuable research tool that permits the proliferation of T cell populations for in vitro analysis and

immunotherapy protocols, has resulted in the publication of thousands of studies. This review focuses on our current understanding of the biological role of IL-2 with an emphasis on the intracellular signaling pathways controlled by IL-2 in T cells, and factors that might influence the biological outcomes of IL-2 signaling.

The IL-2 paradox

The powerful growth factor and mitogenic activities of IL-2 led to the hypothesis that IL-2 would be an essential cytokine that promoted T cell immune responses in vivo. However, this paradigm was challenged by the phenotype of mice with deleted IL-2 alleles: IL-2^{-/-} mice developed normally during the first 3-4 weeks of age (21) but 50% of IL-2^{-/-} mice died within 9 weeks of birth and the remaining mice had anemia, aberrant lymphoproliferation and an inflammatory bowel disease resembling ulcerative colitis (22). A similar autoimmune phenotype was seen in mice with deleted CD25 alleles (23), indicating that this important immunoregulatory role of IL-2 was mediated by the high affinity IL-2 receptor. These data forced a re-evaluation of the role of IL-2 as simply a positive regulator of effector T cell proliferation, and rather suggested that the fundamental role of IL-2 is to suppress immune responses. This concept is reinforced by studies showing that polymorphisms that can alter IL-2 signaling in humans are associated with autoimmune diseases including type 1 diabetes, celiac disease, multiple sclerosis, Grave's disease, and rheumatoid arthritis (24-26).

It was rapidly recognized that the severe immune autoreactivity of IL-2^{-/-} mice is caused by a loss of immunosuppressive CD4⁺ FoxP3⁺ regulatory T (Treg) cells (27, 28). IL-2 is critical for the development of Tregs in the thymus, the regulation, proliferation and maintenance of Tregs in peripheral tissues, and is essential for maintaining the transcriptional program required for Treg function. This includes sustaining the expression of high levels of FoxP3, the key

transcription factor that determines Treg identity (29). IL-2^{-/-} mice have very low numbers of Tregs and those Tregs that are present do not function effectively to control autoimmunity (30).

What about the role of IL-2 in effector T cells? Evaluation of the importance of IL-2 for effector T cell differentiation in vivo has been complicated by the lymphoproliferation induced by the loss of Tregs. However, it is clear that IL-2 influences effector T cell differentiation and is a critical determinant of the fate decisions of antigen receptor activated T cells (31-38). CD4⁺ α/β T cells differentiate following immune activation to produce distinct effector subpopulations that can be distinguished based on their cytokine production, expression of lineage-specific transcriptional “master regulators” and cytokine requirements (39). Th1 cells produce IFNγ, and depend on signals from IL-12 and activation of the transcription factors STAT4 and T-bet. The requirement for IL-12 signals renders Th1 cells dependent on the expression of the IL-12 receptor, which consists of IL-12Rβ1 and IL-12Rβ2. Th2 cells produce the cytokines IL-4, IL-5 and IL-13, and depend on IL-4 signals, activation of STAT6 and GATA3. Thus, Th2 cells depend on the expression of the IL-4 receptor which consists of IL-4Rα and γ_c. Th17 helper cells, which control neutrophil function and epithelial barrier integrity, produce IL-17A, IL-17F and IL-22 and depend on signals from IL-6, in combination with TGF-β, IL-23 and IL-21. These signals result in activation of STAT3 and the expression of the transcription factor RORγt to promote Th17 differentiation. Therefore, expression of the IL-6 receptor, which consists of IL-6Rα and gp130, is important for Th17 differentiation. Finally, T follicular helper (Tfh) cells support B cell-mediated immune responses and germinal center formation in the spleen. Tfh cells require IL-21 and IL-6 for their development, with BCL6 acting as a master regulator of the differentiation of Tfh cells (40).

One fundamental question is what directs CD4⁺ T cells to adopt these different fates? Here it is clear that IL-2 has a role. IL-2 can, thus, promote Th1 and Th2 fate decisions in antigen receptor activated CD4⁺ T cells (35, 36, 41), but suppress the differentiation of Th17 cells (32, 42) and Tfh cells (37, 43) (Figure 1). IL-2 controls CD4⁺ T cell fate by controlling the expression of the key cytokine receptors, transcription factors, chromatin regulators and effector cytokines. For example, IL-2 can induce the expression of Eomesodermin (34, 44), Tbet (Tbx21) (36) and Blimp-1 (34, 45) while suppressing BCL6 (46, 47). Additionally, IL-2 can regulate how cells may respond to other pro-inflammatory cytokines by stimulating the expression of the IL-12R, IL-12R β 1 and IL-12R β 2 chains (36), IL-4R α chain, (41, 48), and suppressing the expression of IL-6R α and gp130 (36).

IL-2 is also important for CD8⁺ effector cells (Figure 1). In response to infection, naïve antigen-specific CD8⁺ T cells proliferate and differentiate into effector cytotoxic lymphocytes (CTL) that make pro-inflammatory cytokines such as IFN γ and acquire the ability to kill infected cells. IL-2 can influence these effector activities in CD8⁺ cells by inducing the expression of IFN γ (49, 50), TNF α and lymphotoxin α (51) and by promoting expression of the cytolytic effector molecules, granzyme B (34, 44, 52) and perforin (34, 52), and promoting effective target cell killing (34, 44). IL-2 also acts to control the cell size of CTL by regulating amino acid uptake and protein synthesis (53-55). The majority of CTL die after pathogen clearance; however, a small number do not terminally differentiate, but switch off active effector functions, and survive as long-lived, self-renewing memory T cells. An understanding of how IL-2 can control the balance in the effector/memory cell fates of CD8⁺ T cells has grown greatly in recent years. High IL-2 signaling drives T cells to become terminally differentiated, short-lived effector cells and promotes the expression of critical cytolytic effector molecules and cytokines by immune activated CD8⁺ T cells (Figure 1); this is because IL-2 induces the expression of Blimp1 whilst

suppressing the expression of molecules associated with central memory effectors, such as BCL6, the IL-7R α chain (CD127) and L-selectin (CD62L) (33, 34, 56). In contrast, low doses of IL-2 signaling can support a memory phenotype in activated CD8⁺ T cells (33, 34, 56) and Tfh-like or memory phenotype in CD4⁺ T cells (38) (Figure 1). Low-dose IL-2 allows BCL6, IL-7R α and L-selectin to be re-expressed, and may not drive expression of high levels of cytolytic molecules, thus, more readily allowing the maturation of memory cells. The low levels of Blimp-1 in cells differentiated in low levels of IL-2 correlate with a more efficient IL-2 production by these cells upon rechallenge (34). This may be important for potentiating the autocrine IL-2 signaling by CD8⁺ cells that is important for CD8⁺ T cell memory responses (57, 58).

IL-2 signal transduction

IL-2 controls diverse T cell biology by binding with high affinity ($K_d \approx 10^{-11}$ M) to the trimeric IL-2R consisting of IL-2R α , IL-2R β and γ_c . The IL-2R β and γ_c dimer can bind IL-2 with lower affinity ($K_d \approx 10^{-9}$ M) but the phenotype of the CD25 knock out mouse indicates that most of the biological functions of IL-2 are mediated by the high affinity IL-2 receptor. Moreover, in early experiments, it became clear that key signaling functions of the IL-2 receptor were mediated by the high affinity receptor. For example, IL-2 receptor triggering causes the Ras GTPase to switch from the inactive GDP loaded to the active GTP bound state: the concentration of IL-2 that activated Ras coincided with those that bound to the high affinity IL-2 receptor (59). GTP bound Ras binds and activates the serine/threonine kinase RAF-1 which directs activity of the MAP kinases ERK1/2 (60). These studies were the first to indicate that some of the biological effects of IL-2 could be mediated by the activation of cytosolic serine/threonine kinases. This concept was reinforced by studies showing that IL-2 controlled the activity of the p70S6 ribosomal kinase (61-63) in a signaling pathway regulated by the

immuno-suppressive drug, rapamycin. This latter result is now known to reflect that IL-2 controls the activity of the nutrient sensing serine/threonine kinase, mammalian target of rapamycin complex 1 (mTORC1) (64).

The challenge, then, was to understand how IL-2 receptors could couple to cytosolic GTPases and serine/threonine kinases. The IL-2 receptor subunits lack intrinsic kinase or other enzymatic activity. However, it was quickly recognized that IL-2 controlled T cell function by regulating the activity of cytosolic tyrosine kinases. It was originally proposed that IL-2 signaled to T cells via SRC family kinases, such as LCK and FYN (65, 66). This hypothesis did not survive experimental tests and it is now known that LCK and FYN have high constitutively activity prior to IL-2 receptor engagement and hence signal independently of IL-2 signals (55). A key break-through in understanding how the IL-2 receptor functions came from the discovery that the IL-2 receptor uses Janus family kinase (JAK) members to induce signal transduction (67-70). The IL-2R β chain binds JAK1, the γ_c chain binds to JAK3 and IL-2 receptor occupancy results in JAK1/3 activation (71). Consequently, JAK1 and/or JAK3 selective kinase inhibitors are powerful tools for blocking IL-2 mediated proliferative responses (72, 73). The importance of JAK3 for γ_c signaling is also clear because the phenotype of humans lacking expression/function of JAK3 is associated with the same type of severe combined immunodeficiency (SCID) found for humans with γ_c loss of expression/function (73, 74). There is however, still some debate over the role of the kinase activity of JAK3 versus its role as a signaling scaffold in mediating IL-2-IL-2R transduction (75).

The discovery of the IL-2-JAK connection rapidly led to the discovery that IL-2 induced the tyrosine phosphorylation and DNA binding of a high molecular weight protein of the Signal Transducer and Activator of Transcription (STAT) family (76). This was subsequently mapped

to key tyrosine residues close to the c-terminus of STAT5 proteins, STAT5A Y694 and STAT5B Y699 (77, 78) (Figure 2A). In addition to the tyrosine phosphorylations, IL-2-induced serine phosphorylation of STAT5 has been proposed to regulate STAT5 transcriptional function (78). Serine residues increased in phosphorylation in response to IL-2 have been mapped to STAT5A S127 and/or S128 (55) (Figure 2A), sites that may influence the transcriptional activity of the protein (79). IL-2 can also regulate other STATs and induce phosphorylation of STAT3 on Y705 and S727 (77, 80) (Figure 2A) and STAT1 phosphorylation on Y701 (15). The model for STAT5 activation is that activation of JAK1 and JAK3 induces a number of tyrosine phosphorylations in the IL-2R β chain including Y338, Y392 and Y510 in the human receptor (Y341, Y395 and Y498 in the mouse IL-2 receptor) (15) (Figure 2A). The phosphorylation of IL-2R β at Y395 and Y498 allows the recruitment of STAT5A and STAT5B transcription factors via their SH2 domains and their subsequent tyrosine phosphorylation (Figure 2A); following phosphorylation, STAT5 proteins dissociate from the IL-2 β subunit and form dimers, through the head-to-tail association of the SH2 domains with the phosphorylated phosphotyrosine sites. This induces their transcriptional activation and translocation to the nucleus. These STAT dimers can bind to gamma interferon activated sequence (GAS) DNA sequences (TTCN₃GAA) to induce transcription. The STAT5 dimers can also form tetramers through interactions between residues (I28, F81 and L82) in their N-terminal regions. These STAT5 tetramers bind to pairs of GAS motifs separated by a linker of 6-22 nucleotides (51). Mutational studies demonstrate that STAT5 dimers and tetramers are both important for IL-2-induced gene expression (51).

IL-2 activation of STAT5 mediated transcriptional programs is undoubtedly key to the biological actions of IL-2 (28, 73, 81-83). The critical importance of STAT5A/B-mediated transcriptional processes has been highlighted by studies of knockout mice. STAT5A/B-

deficient mice fail to generate Tregs (84-86) demonstrating the crucial role for IL-2-JAK1/3-STAT5 in Treg development. In addition, immune activated peripheral T cells from STAT5A/B knockout mice are unable to proliferate (81, 87). Moreover, in humans, loss-of-function in STAT5B is associated with a loss of Tregs (88, 89). STAT5A and STAT5B are thus critical mediators of IL-2 signals. Analyses of STAT5 binding sites using chromatin immunoprecipitation (ChIP) in CD4⁺ T cells have identified that a number of essential lineage-defining transcription factors are direct STAT5A and STAT5B target genes. These include *FoxP3* (85, 90), *Tbx21* (T-bet) (36), *Maf* (41, 91) and *Gata3* (41). STAT5 can additionally bind to the *Il2ra* gene (90), *Il12rb2* (36) and *Il4ra* (41), correlating with the ability of IL-2 to induce the expression of these genes, sustain its own signaling and render T cells susceptible to inputs from other cytokines to promote the differentiation of Th1 and Th2 cells. Another STAT5 target gene is *Socs3* (41, 51, 92) – a protein that negatively regulates IL-2 signals. However, not all STAT5-binding sites are associated with increases in gene expression: STAT5 binding to the *Il17a-Il17f* locus is repressive, thereby inhibiting STAT3-mediated transcription of the *Il17* gene (42) and suppressing Th17 cell differentiation. Similarly, STAT5 has been shown to bind *Bcl6* gene in hematopoietic cell lines (93) and in CD4⁺ T cells (47, 94) correlating with repressed expression of the gene. The mechanisms by which STAT5 may regulate gene expression in T cells, such as the recruitment of RNA polymerase II, co-activating or co-repressing proteins, or chromatin remodelling enzymes, are still to be elucidated. The amino acid sequence of STAT5A and STAT5B shows over 90% similarity, and homodimers of each of these two proteins recognize very similar GAS motifs (81). While differences in STAT5A and STAT5B genes targets have been suggested in T cell subsets (41, 90), it is likely this may reflect differences in their relative expression in vivo rather than functional differences (94).

IL-2 signaling beyond STAT5

Although activation of STAT5 is important for IL-2 signaling, experiments with constitutively active mutants have found active STAT5 is not sufficient to mimic the effects of IL-2 on T cell biology (95). It is, therefore, clear that IL-2 signaling networks extend beyond STATs and include other signaling networks. As discussed, IL-2 drives the accumulation of active, GTP-bound Ras GTPases (59). The key pathway for IL-2 control of Ras is mediated by the adapter SHC which is tyrosine phosphorylated on Y317 (Y313 in mouse) in response to IL-2 receptor engagement (96) (Figure 2A). Tyrosine phosphorylated SHC then forms a complex with the SH2 domain of the adapter GRB2 which binds constitutively via its tandem SH3 domains to the Ras guanine nucleotide exchange protein SOS (Son of Sevenless) (97) (Figure 2A). This complex catalyzes the exchange of GDP for GTP on Ras, thereby allowing Ras to accumulate in its GTP bound state and activate the Raf-ERK MAP kinase cascade (Figure 2A).

Another important serine threonine kinase for IL-2 signal transduction is the serine/threonine kinase, mammalian target of rapamycin (mTOR) (43, 64) (Figure 2B). The mTOR kinase is a constituent of two different kinase complexes, mTORC1 and mTORC2. The core subunits of mTORC1 consists mTOR, RAPTOR and mLST8, while the core subunits of mTORC2 are mTOR, RICTOR and mLST8 (98). The significance of the mTOR complex for T cell biology is evidenced by the importance of the mTORC1 inhibitor rapamycin as a T cell immunosuppressant. Moreover, CD4⁺ T cell differentiation into Th1 cells is inhibited by T cell specific knockout of the mTOR kinase (99), Rheb (100) and RICTOR (100, 101), thus, implicating both mTORC1 and mTORC2 complexes in Th1 development. Interestingly, Th2 cells appear to depend on mTORC2 signaling as Rheb^{-/-} T cells were capable of differentiating into Th2 cells (100), but mTOR^{-/-} and RICTOR-deficient T cells could not (99, 101, 102). In

contrast, IL-2-mediated activation of mTORC1 has been shown to be involved in IL-2 suppression of Tfh differentiation (43).

In effector CD8⁺ cytotoxic T cells, IL-2 induces and maintains a robust activation of mTORC1 to control IL-2-induced metabolic and transcriptional programs (53, 54, 64). Hence mTORC1 activity appears to play an important role in shaping the CTL proteome to control energy-generating metabolic process (17, 64). Loss of mTORC1 signaling, thus, causes CTL to decrease expression of glucose transporters (17, 64) and glycolytic enzymes (Figure 2B) and, hence, to decrease their rate of glycolysis while maintaining oxidative phosphorylation pathways (17). In addition, mTORC1 activity is required to sustain the expression of proteins required for cholesterol biosynthesis, such as 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGCR) (17) (Figure 2B). Interestingly, mTORC1 activity may suppress some metabolic pathways in CTL; rates of glutaminolysis are increased in mTORC1 inhibited, i.e. rapamycin-treated, CTL (17).

One important role for mTORC1 activity in CTL is to control the expression of effector molecules including perforin, granzymes and IFN γ (17). Moreover, a key differentiation step for effector CTL is to switch off the expression of chemokine receptors and adhesion molecules, such as CD62L, CCR7 and S1P1, that direct naïve and memory T cells to secondary lymphoid organs (103). This part of the CTL transcriptional program is essential to prevent CTL entering secondary lymphoid tissue and hence promotes CTL trafficking to peripheral tissues where they mediate destruction of pathogen infected cells. The inhibition of mTORC1 in IL-2 maintained CTL causes the cells to retain CD62L and CCR7 expression and hence retain the capacity to home to secondary lymphoid tissue (103).

How does IL-2 regulated mTORC1 control CTL function? Here, a crucial molecule is the transcription factor HIF1 α . IL-2 sustains expression of HIF1 α in CTL via an mTORC1 dependent pathway (Figure 2B). HIF1 α forms a transcriptional complex with ARNT/HIF1 β to regulate gene expression. HIF1 controls a subset of mTORC1-regulated genes, including glucose transporters and of genes involved in glycolysis and pyruvate metabolism as well as the effector molecule perforin (64) (Figure 2B). Not all effector molecules are regulated by HIF1, as HIF1 knockout CTL are still capable of producing IFN γ and expression of FAS ligand and lymphotoxin α (64). However, the HIF1 pathway is critical for mTORC1 repression of the expression of the LN homing molecules, CD62L, CCR7 and S1P1 (64). Immune activated HIF1 null CD8⁺ T cells, thus, retain the migratory pattern of naïve T cells to secondary lymphoid tissue (64).

How is mTORC1 activity sustained by IL-2 in CTL? It is known that IL-2 control of mTORC1 activity is dependent on JAK kinase activity (55). Additionally, mTORC1 activity in IL-2 stimulated T cells is regulated by nutrient availability. Indeed, a constant source of amino acid signaling via leucine, glutamine and arginine is required for mTORC1 activity (98), and is a prerequisite for mTORC1 activation in T cells (54) (Figure 2B). mTORC1 activity in CTL is also highly dependent of sustained availability of glucose (104). CTL are able to sense glucose levels via the AMP activated protein kinase (AMPK). If the glucose supply to T cells is limited, AMPK is activated, triggering a negative feedback pathway to restrict mTORC1 activity (105) (Figure 2B). The high sensitivity of mTORC1 activity to nutrients makes it probable that the ability of IL-2 to maintain amino acid uptake, and possibly glucose uptake (53, 54, 64), are key drivers of mTORC1 activity. In this respect, one key IL-2-JAK1/3 maintained molecule in this nutrient-sensitive signaling pathway is the system L amino acid transporter, SLC7A5 (Figure

2B). This transporter sustains the uptake of leucine and other large neutral amino acids (54), and is the most highly expressed IL-2 regulated leucine transporter in CTL (17).

Following the advancement of quantitative mass spectrometry technologies, a number of studies have taken unbiased approaches to investigate the phosphorylation pathways induced in cells in response to IL-2 activation on a global scale. Studies in KIT225 cells (106-109), F15R-Kit cells (110) and in primary mouse CTL cultures (55) have made it possible to evaluate the diversity and true extent of phosphorylation networks influenced by IL-2 signaling (Figure 3). The picture of signaling that emerged from the primary CTL phosphoproteomics study (55) exposed the striking number and range of protein phosphorylations that can be influenced by IL-2; out of 6458 phosphorylation sites identified in CTL, IL-2 increased the phosphorylation of 707 and decreased 224 phosphorylations. These data highlight how much of IL-2 signaling is under-appreciated, particularly that IL-2 can both positively and negatively influence the phosphorylation status of proteins in a cell. The majority of the phosphorylations influenced by IL-2 were phosphoserine and phosphothreonine sites, a number of which were inversely regulated by treating IL-2 maintained CTL with the JAK1/3 inhibitor Tofacitinib (55). Thus, these data give a perspective of the diverse phosphoserine and phosphothreonine signals that IL-2-JAK1/3 signaling can modulate. The phosphoproteins regulated by IL-2 included adaptor proteins, proteins involved in processes in the nucleus, the actin and microtubule cytoskeleton and membrane trafficking (55, 106-110) (Figure 3). The web of phosphorylation networks uncovered by such global studies demonstrates how multiple aspects of T cell biology may be synchronized by IL-2. In addition, they reveal how IL-2 may control the differentiation and fate decisions in T cells. Of particular note was the ability of IL-2 to regulate the phosphorylation of histone modifiers and chromatin-remodeling enzymes (55, 109) (Figure 3), thus, indicating how IL-2 can control gene expression and may influence memory cell recall

responses of T cells. Such data also emphasize that IL-2 may direct the protein expression in T cells separately from instigating transcriptional programs: IL-2 can regulate the phosphorylation of mRNA binding proteins such as YBX1 and LARP1 (55), that can control the stability of mRNAs and direct which are translated into proteins (Figure 3). Additionally, IL-2 regulated the phosphorylation of components of the cellular translational machinery, correlating with the ability of IL-2 to control the rate of protein synthesis in CTL (55) (Figure 3). These phosphoproteomic studies have given unparalleled insights into the proteins that are influenced by IL-2 signaling. Thus, future phosphoproteomics and proteomics analyses will powerfully accelerate understanding of the molecules that are regulated by IL-2 in different cell types and permit in-depth dissection of how IL-2 can be tailored to regulate both pro- and anti-inflammatory T cells.

Indeed, an important aspect to consider is how IL-2 may downregulate/switch-off signaling pathways in T cells. Some potential pathways linking IL-2R occupancy to tyrosine phosphatases have been characterized: SHC-GRB2 complexes associated with the IL-2R may allow the recruitment of the adaptor, GAB2, which, in turn, may recruit the protein tyrosine phosphatase SHP-2 (PTPN11) to the IL-2 receptor (111). SHP-2 has been shown to be phosphorylated in response to IL-2 (112): this may lead to molecular re-arrangements that induce the activation of its phosphatase activity (113). However, SHP-2 may additionally act as an adaptor molecule in IL-2 signal transduction and, despite its phosphatase activities, has been implicated in positively regulating IL-2 signaling (114). Interestingly, SHP-1, a phosphatase related to SHP-2, has also been found to associate with the IL-2 receptor complex, and may influence the tyrosine phosphorylation of receptor components (115) in a negative feedback loop to terminate IL-2 receptor signaling. IL-2 via STAT5 can also stimulate the expression of the SH2-domain-containing E3-ubiquitin ligases, CIS, SOCS1 and SOCS3 (51).

These proteins can either associate with the IL-2R β to block interaction sites, directly inhibit JAK1 activity, and/or mediate proteosomal degradation of bound proteins (116). Therefore, the extent of activation of such inhibitory pathways may also impact on IL-2 signaling outcomes in T cells.

IL-2 and PI 3-kinase signaling

A key signaling pathway in T cells is mediated by the lipid, phosphatidylinositol (3,4,5) trisphosphate (PI(3,4,5)P₃). Cellular levels of PI(3,4,5)P₃ are controlled by the balanced activity of kinases and phosphatases. Class I phosphatidylinositol 3-kinases (PI3Ks) phosphorylate the 3-position of the inositol ring of phosphatidylinositol (4,5) bisphosphate (PI(4,5)P₂) to produce PI(3,4,5)P₃. The class I PI3Ks comprise of a 110 kDa catalytic subunit and an adapter regulatory subunit: there are four catalytic isoforms (α , β , γ , δ) and three adapter subunits (p85 α , p85 β , p55 γ). In mature T cells, the majority of PI(3,4,5)P₃ is produced by the actions of the p110 δ catalytic subunit in a complex with p85 α or p85 β (117). PI(3,4,5)P₃ can then be metabolized in one of two ways; either via the lipid phosphatase, PTEN (phosphatase and tensin homologue deleted on chromosome 10), which removes the phosphate group from the 3-position of PI(3,4,5)P₃ to produce PI(4,5)P₂ or via the SHIP family phosphatases (SHIP-1 and SHIP-2), which de-phosphorylate PI(3,4,5)P₃ to PI(3,4)P₂ (117).

PI(3,4,5)P₃ can organize signaling transduction in cells by interacting with pleckstrin homology (PH) domains in proteins and localizing these proteins to membranes. Proteins that bind to PI(3,4,5)P₃ in T cells include the serine/threonine kinases phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB also known as AKT); Tec family tyrosine kinases; and guanine nucleotide exchange proteins for Rho family GTPases (118). In addition to establishing the localization of proteins, the binding of PH domains to PI(3,4,5)P₃ can initiate conformational changes in proteins: for example, the catalytic activity of AKT is allosterically

regulated by PI(3,4,5)P₃ (119). The activities of PI(3,4,5)P₃-binding serine/threonine kinases play an important role in the differentiation of CTL. PDK1 controls the activity of mTORC1 and T cell metabolic programs (64). Additionally, PDK1 controls the activity of AKT in IL-2 maintained CTL: PI(3,4,5)P₃ facilitates the co-localization of AKT and PDK1, allowing PDK1 to phosphorylate AKT on T308, thereby activating AKT (120). The PDK1/AKT axis is key for effector T cell differentiation because of ability of AKT to phosphorylate and enforce the nuclear exclusion, and, consequently, repress the activities of the FOXO1/3 transcription factors. This regulation of FOXO1/3 is an important governor of T cell fate because FOXO1/3 proteins induce the expression of lymphoid homing trafficking molecules while simultaneously repressing the expression of effector molecules in CTL, thus directing effector/memory CD8⁺ T cell differentiation (121-123). Therefore, the activation of PI(3,4,5)P₃-AKT is pivotal for effector T cell differentiation (121, 124). However, although loss of PI3K-p110 δ activity is detrimental to T cell development, constitutive activation of PI3K-p110 δ also impairs immune function (117, 125-127). It is increasingly clear that the activation of PI3K-p110 δ must be finely balanced: determining how cells appropriately regulate this pathway is key to understanding the molecular mechanisms that control immune homeostasis. However, understanding the regulation of this pathway by IL-2 has been challenging.

In the 1990s, it was proposed that activation of the PI3K-AKT signaling pathway was regulated by IL-2 (62, 63, 128). This model stemmed from observations that the p85/PI3K-p110 could be recruited into the IL-2R signaling complex (129-131). The p85 regulatory subunit of PI3K contains an SH2 domain that can bind with high affinity to phosphorylated tyrosines, normally within the motif YXXM. The pY392 (mouse Y395) in IL-2R β has been implicated in recruiting the p85 adaptor subunit of PI3K to the IL-2R complex (129). However, this site does not fit the consensus YXXM binding motif for the p85 SH2 domain. Interestingly, other studies

indicated that p85 may be recruited to the receptor by non phosphotyrosine-mediated interactions with IL-2R β and JAK1 (130). An alternative mechanism by which p85 may be recruited to the receptor is via interactions with tyrosine phosphorylated GAB2, which can associate with the IL-2R via SHC-GRB2 complexes (131).

One approach to investigate the role of PI3K-AKT signaling in T cells examined the impact of overexpressing plasma membrane-localized PI3K-p110 or constitutively active AKT in the IL-2R-expressing T cell tumour line, KIT225 (63). These experiments indicated that active PI3K or AKT could partially mimic IL-2 and regulate the transcription factor E2F, which is key for T cell cycle progression. Such results demonstrated what the PI3K-AKT signaling pathway was able to do in T cells, but they did not prove that the pathway was regulated by IL-2. Other experiments investigated the impact of overexpressing a mutated p85 subunit that was unable to interact with the p110 catalytic subunit on IL-2 signaling. The rationale for these experiments was that the mutated p85 subunit would associate with p85 docking sites in the IL-2R or adaptor proteins but would fail to recruit p110 δ to the plasma membrane and, hence, function as a dominant negative PI3K. Overexpression of this mutant p85 blocked IL-2 control of cell cycle progression (62); however, it is now clear that this p85 construct would not be selective for PI3K docking sites and when overexpressed would have the potential to block multiple signaling complexes, not just activation of PI3K.

Arguably, the most persuasive experiments to indicate that IL-2 activated PI3K in T cells came from studies using the PI3K inhibitors, LY294002 and wortmannin. For example, LY294002 blocked IL-2 mediated cell cycle progression of KIT225 cells (62) and IL-2-induced cell growth of CD8⁺ T cells (53), thus supporting a role for PI3K signaling in the IL-2 regulatory program. Further analysis of the biochemical pathways regulated by LY294002 and

wortmannin linked loss of PI3K-AKT signals to the activation of FOXO transcription factors and the inhibition of mTORC1 (132, 133). As a result, it has become a paradigm that IL-2 signaling activates the PI3K-AKT pathway to inhibit FOXO1/3 transcription factors and activate mTORC1 and its substrate, p70S6K (p70 ribosomal S6 kinase) in T cells. Consequently, many subsequent studies have used the output of p70S6K, as measured by the magnitude of the phosphorylation of the ribosomal protein, S6, as a surrogate method assess the accumulation of PI(3,4,5)P₃ or activation of the PI3K-AKT signaling pathway in T cells. However, we now know that using LY294002 and wortmannin to identify downstream targets of the PI3K pathway is flawed as these inhibitors have numerous off-target effects. Both inhibitors directly block mTOR catalytic activity and LY294002 is also a potent inhibitor of the PIM family of serine/threonine kinases (134, 135). Therefore, decreased phosphorylation of mTORC1 substrates in response to LY294002 or wortmannin can occur via direct inhibition of mTOR activity, and may not reflect any role for PI3K-AKT signaling. Moreover, any impact of LY294002 on T cell biology would reflect the impact of combined inhibition of PI3Ks, mTOR and PIM kinases and should be interpreted cautiously.

A further complication in evaluating PI3K-AKT signaling downstream of the IL-2R has been the assessment of AKT phosphorylation itself: many studies use an antibody that detects AKT S473 phosphorylation, rather than an antibody that recognises the activating T-loop phosphorylation site in AKT, phospho-T308. AKT S473 is phosphorylated by the mTORC2 complex and, unlike phospho-T308 is not essential for AKT activity (120). Moreover, loss of AKT S473 phosphorylation does not necessarily result in a decrease in AKT T308 phosphorylation or correlate with a decrease in cellular levels of PI(3,4,5)P₃ (17, 136). Thus, monitoring AKT S473 does not inform on AKT activity or cellular levels of PI(3,4,5)P₃. Therefore, to assess the PI3K-AKT signaling pathway accurately, PI(3,4,5)P₃ must either be

measured biochemically (137) or monitored using imaging techniques that follow the membrane accumulation of fluorescently-tagged, and PI(3,4,5)P₃-specific, PH domain probes (138, 139). Any assessment of AKT activity should be made by quantifying AKT T308 phosphorylation. If a substitute measure of AKT activity is required, the phosphorylation and nuclear exclusion of the direct AKT substrates FOXO1/FOXO3 are likely to be more reliable than those downstream of mTORC1 signaling.

In view of these caveats, what, then, is known about the role of PI3K-AKT in the context of T cell biology and IL-2 signaling? Here, key advances have been made following the development of small molecules with improved inhibitor specificities, and by looking at T cells that have wild type p110 δ alleles replaced with catalytically inactive p110 δ^{D910A} alleles (64, 121). For example, IC87114 is a highly specific inhibitor of PI3K-p110 δ and AKTi is an inhibitor of AKT that, rather than targeting its catalytic pocket, interferes with the PI(3,4,5)P₃-mediated recruitment of AKT to the plasma membrane (140). Studies have shown that the IL-2-mediated proliferative expansion of effector CD8⁺ CTL is normal in the presence of AKTi or IC87114 (121). Both IC87114 and AKTi block the phosphorylation of AKT T308 and the phosphorylation of the AKT substrates, FOXO1 and FOXO3, demonstrating that they inhibit the activation of AKT. However, a critical finding using these inhibitors was that neither compound blocked the phosphorylation of the mTORC1 substrate, p70S6K1, nor of its substrates 4EBP1 and S6 phosphorylation (64). Thus, these data indicate that PI3K-AKT activity is not responsible for maintaining mTORC1 activity in CTL. Further supporting this model, CTL expressing the catalytically inactive p110 δ^{D910A} mutant did not activate AKT but showed normal proliferative responses and mTORC1-dependent phosphorylation of S6K1 and S6 (64, 121). Additionally, loss of p110 δ or AKT activity had no impact on the ability of IL-2 to sustain the expression of the glucose metabolic pathways mediated by HIF1 α downstream

of mTORC1 (64, 121). Conversely, deletion of PTEN, which increases cellular PI(3,4,5)P₃ and can drive the activation of AKT, is not sufficient to activate mTORC1 and mTORC1-dependent glucose metabolism (141). Together, these data demonstrate that IL-2 can activate and sustain mTORC1 activity without the need for activation of PI3K or AKT.

In terms of understanding the link between IL-2 signaling and the accumulation of PI(3,4,5)P₃ levels in CTL, direct measurements of cellular lipids has confirmed that PI(3,4,5)P₃ levels decrease in IL-2 maintained CTL following PI3K-p110 δ inhibition (55). However, no measurable effect on PI(3,4,5)P₃ levels was detected following treatment of IL-2 maintained CTL with a JAK1/3 inhibitor or in response to IL-2 removal. It was also clear that IL-2-JAK1/3 activity did not regulate FOXO1 phosphorylation and nuclear exclusion in CTL (55). Rather, cellular levels of PI(3,4,5)P₃, the activity of AKT and the phosphorylation and nuclear exclusion of FOXO transcription factors are controlled by IL-2 independent signaling networks mediated by SRC family kinases, LCK and FYN (55). Although LCK activity is associated with antigen receptor signaling in T cells, the activity of LCK is constitutive and not controlled by antigen receptor occupancy (142). Thus, IL-2 stimulated effector T cells have high levels of activity of the SRC family kinases p56LCK and FYN but the activity of FYN and LCK is intrinsic and is not controlled by IL-2. Hence, while the original IL-2 signal transduction models placed PI(3,4,5)P₃-AKT pathways downstream of the IL-2 receptor and JAKs, these models need to be revised to include our new understanding that the PI(3,4,5)P₃-AKT pathway may be maintained in T cells independently from IL-2-JAK1/3-signaling (Figure 4). Indeed, such findings may explain why AKT activity is not maintained or induced in IL-2-dependent T subsets (143). This key revision of IL-2 signal transduction highlights that IL-2 signals within a context of other “pre-organized” signal transduction pathways (Figure 4). Indeed, the role of these pre-existing pathways is being increasingly appreciated. Another kinase that, like LCK

and FYN, is constitutively active in CTL is GSK3. The activity of GSK3 controls the expression of MYC in IL-2 maintained CTL (144). Similarly, the nuclear exclusion of the chromatin regulator of HDAC7 is determined by the constitutive activity of serine/threonine kinases (145). Thus, the output of IL-2 signaling in individual T cell subsets may be defined by extrinsic factors including the expression levels and activity of constitutively active kinases.

Defining IL-2 signaling in different T cell subsets

A defining characteristic of IL-2 is that its biological activities appear to be highly tailored to induce cell-type specific responses. Hence a key role for IL-2 in Treg cells is to support their differentiation and homeostasis while in effector T cells IL-2 stimulates differentiation, proliferation and cell growth. Moreover, these activities appear to be exclusive, as, for example, IL-2 does not induce rapid proliferation in Tregs (143). Most studies that have dissected IL-2 signaling have been performed in antigen receptor-activated CD4⁺ or CD8⁺ T cells where IL-2 is a potent mitogen and growth factor. How, then, are the diverse signaling pathways that have been mapped to IL-2 signaling in these effector cells functioning in different T cell subsets? For example, as Tregs and effector cells have very different phenotypes, and since IL-2-activated signals, such as mTORC1, can have opposing impacts on their development, it is unlikely that IL-2 activates and sustains identical pathways in Tregs versus effector cells.

A possible answer is that IL-2 induces different signals in different T cell subsets. This could occur because of different thresholds of IL-2 signals received by cells depending on the concentrations of IL-2 in their environment and the duration for which cells are exposed to them. In vivo, the IL-2 gradients are extremely localized (146) so only certain cells will receive high concentrations of IL-2. This could dictate biological responses to IL-2 as early studies revealed that T cells can “count” the IL-2 signals that they receive. Thus, at least some IL-2

responses are quantal (all-or-none) rather than analogue. For example, single cell analysis of the DNA synthesis in response to a saturating dose of IL-2 compared to a half-saturating dose of IL-2 revealed that the higher concentration of IL-2 induced cell cycle progression more rapidly. Therefore, in situations where the IL-2 is limiting, it takes longer for the required number of activated receptors to accumulate (147-150).

Another likelihood is that signals induced by IL-2 will be modified by other signaling inputs. Many studies are now beginning to emphasize that IL-2 signaling does not act in isolation, but rather in a backdrop of other signaling pathways which can integrate with, and modulate, IL-2 responses. One factor that will influence this is the proteome of the T cell, such as the expression levels of kinases, phosphatases, adaptor proteins and other signaling molecules that will influence signal transduction by IL-2. Examples of this include the levels of the lipid phosphatase PTEN, which has been shown to be a critical determinant of AKT signals in Tregs (143, 151), and the levels of LCK and FYN, which may influence how IL-2-dependent T cell subsets accumulate PI(3,4,5)P₃. Similarly, other signals received by T cells may influence the IL-2 response. For example, Tregs require continuous signals from antigen receptors (152, 153). In addition, following immune challenge, the specific cytokine milieu induced by innate immune cells may influence the outcomes of IL-2 signaling responses.

One final consideration about IL-2 signaling is that it has largely been dissected based on understanding IL-2R-induced phosphorylation networks. However, other types of regulatory modifications on proteins may impact on IL-2-mediated signaling pathways too. For example, recent studies in cell lines suggest that IL-2R occupancy can induce protein acetylation to regulate signaling (154). Additionally, the ability of IL-2 to potentiate glucose and glutamine uptake in certain T cell subsets may also permit or stimulate the modification of proteins by O-

GlcNACylation. This could be important in defining phosphorylation events as O-GlcNAC modifies proteins on serine and threonine residues. For example, O-GlcNacylation of MYC on T58 blocks the phosphorylation that licenses MYC for degradation. Thus, O-GlcNacylation was found to be important for stabilized MYC expression in CTL (155). In this respect, another factor that may shape IL-2 signaling responses may be microenvironmental nutrient levels, such as glucose and amino acids. Similarly, environmental oxygen levels, through modulating protein proline hydroxylation (156), may add a further level of regulation to IL-2 signals. Therefore, a key objective, and challenge, for further understanding IL-2 biology will be to determine how IL-2 signaling may be influenced by other immunoregulatory factors.

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Figure Legends

Figure 1

IL-2 biology: IL-2 positively influences the homeostasis and development of a number of different T cell lineages in the immune system. Expression of the IL-2 receptor is restricted to antigen-activated T cells, thereby guaranteeing the specificity of IL-2 activities. This includes the anti-inflammatory T_{regs} and pro-inflammatory CD4⁺ and CD8⁺ effector cells. These T cell subsets have extremely diverse characteristics and biological roles. IL-2 inhibits the differentiation Th17 and Tfh cells, thus making IL-2 an important regulator of T cell lineage commitment. In addition, the levels of IL-2 signaling can define the effector/memory fates of CD4⁺ and CD8⁺ T cells: high levels of IL-2 favor the development of short-lived effector cells, while low levels of IL-2 signaling promotes the differentiation of memory T cells.

Figure 2

Signaling downstream of the IL-2 receptor: A) JAK-STAT signal transduction. The IL-2R β chain couples to JAK1, and the γ_c chain couples to JAK3. IL-2R occupancy results in the activation of the JAKs and the tyrosine phosphorylation of IL-2R β and γ_c chains (yellow circles). The tyrosine phosphorylations in the IL-2R β are best characterized in terms of the signaling complexes they co-ordinate; the phosphorylation of Y395 and Y498 (in the murine IL-2R β) permit the recruitment of STAT5A, STAT5B and STAT3, while the phosphorylation of Y341 allows recruitment of SHC1. Following their recruitment to the receptor, STAT5 proteins and SHC1 are tyrosine phosphorylated. Tyrosine phosphorylation of STAT5 permits the dimerization, nuclear translocation and, thus, STAT5-mediated transcription. Tyrosine phosphorylation of SHC1 allows the recruitment of GRB2 and SOS to facilitate GTP loading of Ras and activation of the classical Raf-ERK MAP kinase cascade. STAT proteins are also

phosphorylated on serine residues (peach circles) in response to IL-2 signaling. While the STAT5 serine kinase is unknown, the STAT3 proteins are phosphorylated in response to MAP kinase signaling. B) Metabolic pathways activated downstream of the IL-2 receptor. IL-2 sustains the expression of MYC and activation of the mTORC1 signaling complex to maintain the uptake of nutrients such as iron and glucose. IL-2 also sustains the expression of the amino acid transporter, SLC7A5, which transports many of the essential amino acids, such as leucine, into cells. Amino acids are required to sustain mTORC1 activity and fuel translation. Glucose metabolism, via glycolysis and oxidative phosphorylation (OxPhos), sustains ATP generation, and high ATP:ADP and ATP:AMP ratios, thus preventing the activation of AMPK, which inhibits mTORC1. Protein synthesis is essential for maintaining the expression of proteins, such as MYC, with high turnover rates. Through the mTORC1 pathway, IL-2 can also sustain glycolytic metabolism and other biosynthesis in CTL to support cell proliferation, growth and the expression of effector molecules.

Figure 3

The phosphorylation networks of IL-2 signaling in effector T lymphocytes: Global phosphoproteomic studies have revealed the range of tyrosine (yellow circles) and serine/threonine phosphorylations (peach circles) regulated following IL-2 stimulation of CTL. These phosphorylations are on diverse proteins involved in multiple aspects of T cell biology and include scaffold proteins; proteins that can direct gene expression; and cytoskeletal regulators. These comprehensive phosphoproteomic studies highlight that IL-2 signaling is much more than JAK1/3-STAT5 and re-inforce the importance of serine/threonine kinase pathways in IL-2 responses.

Figure 4

Revised model for IL-2 signal transduction: IL-2 signaling outputs are likely to be modulated by other cellular signals, such as “pre-organized” phosphorylation networks that do not depend on IL-2. Constitutive LCK/FYN activity has the potential to influence the phosphorylation of a number of signaling molecules in T cells, and may be a key controller of the accumulation of PI(3,4,5)P₃ in IL-2-dependent T cells. Levels of PI(3,4,5)P₃ in cells depend on the balance in activity of PI3K, which phosphorylates PI(4,5)P₂ to generate PI(3,4,5)P₃ and the PI(3,4,5)P₃ phosphatases, PTEN and SHIP1/2. PI(3,4,5)P₃ can co-ordinate the localization of a number of proteins, including PDK1 and AKT. Phosphorylation of T308 in the activation loop of AKT is induced by PDK1 and is coordinated by PI(3,4,5)P₃ and mTORC2-mediated phosphorylation of AKT S473. Activated AKT may then go on to phosphorylate and inhibit FOXO family transcription factors, to influence transcriptional processes in T cells. However, PI3K-AKT activity in CTL is not a prerequisite for mTORC1 activity, and sustained activation of this complex is dependent on glucose and amino acid levels that can be regulated by IL-2 and environmental nutrient availability.

Figure 1

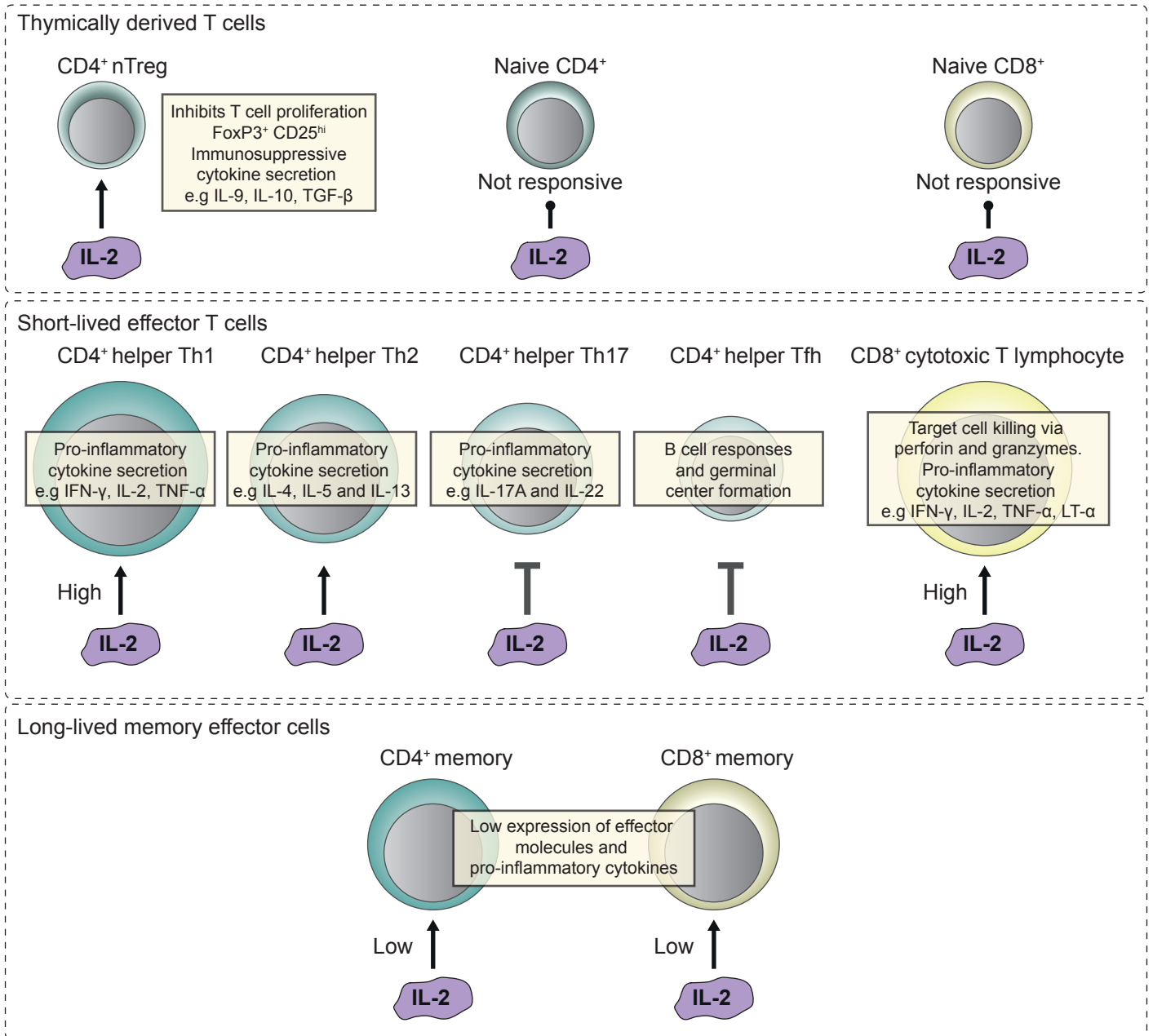


Figure 2

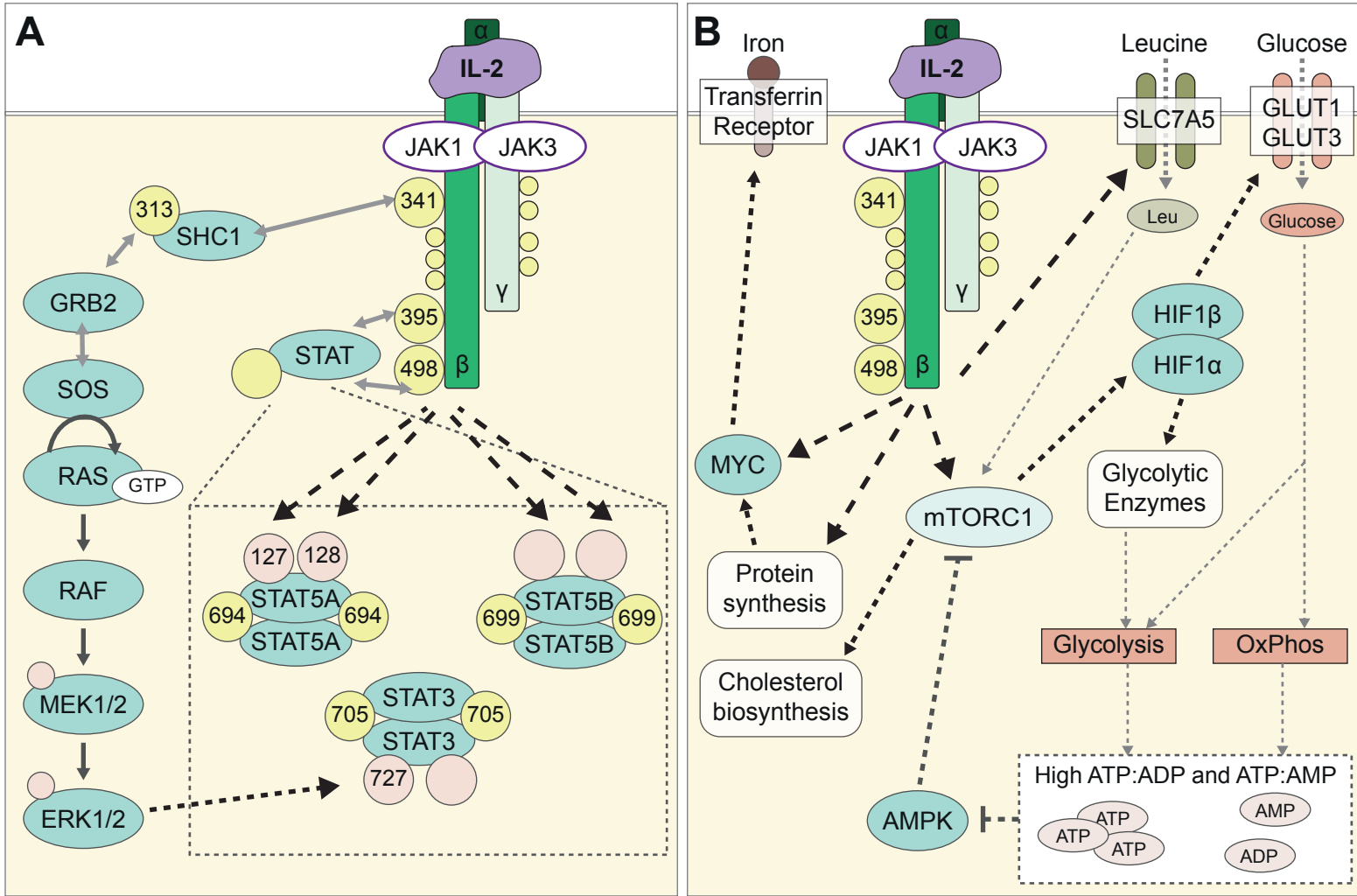


Figure 3

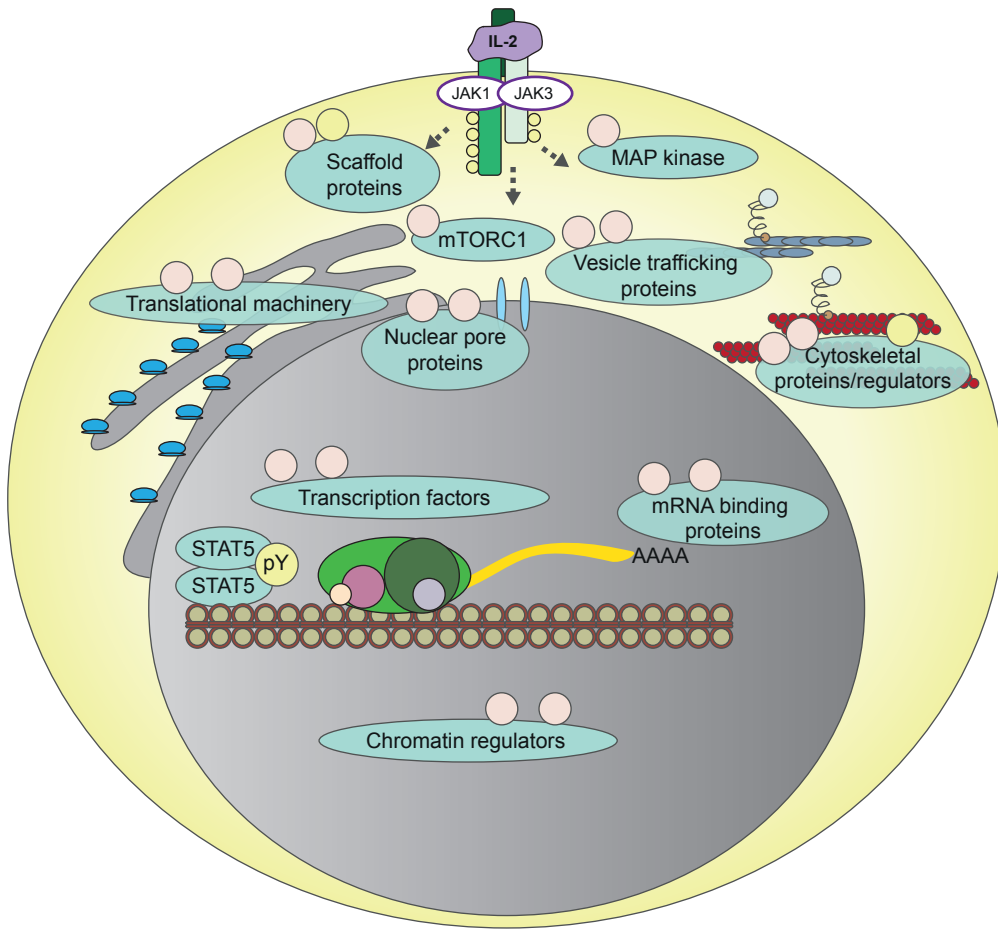


Figure 4

