ABSTRACT

COMFORT, KRISTEN KRUPA. Intracellular Signaling Networks in the Immune Response: Pathways Activated by Interleukin-2 and Interleukin-4 Receptors and their Roles in T Cell Proliferation. (Under the direction of Dr. Jason M. Haugh).

Cells sense and respond to chemical and physical stimuli through signal transduction pathways, which mediate cell proliferation, differentiation, migration, and survival. The cytokines interleukin-2 (IL-2) and interleukin-4 (IL-4) are key regulators of the adaptive immune system, particularly influencing the clonal expansion and differentiation of T cells. At least in culture, both synergistic and antagonistic effects of IL-2 and -4 co-stimulation have been reported; the antagonism, when observed, is thought to arise from the utilization of a common subunit shared by IL-2 and IL-4 receptors. We have sought to characterize IL-2 and IL-4 signaling at the level of intracellular pathways activated by these receptors. IL-2 receptors are known to activate the Ras/extracellular signal-regulated kinase (Erk) and phosphoinositide (PI) 3-kinase pathways as well as the STAT5 transcription factor. IL-4 is unique among cytokines in that it does not activate Ras/Erk; it does activate PI 3-kinase/Akt as well as a distinct STAT, STAT6.

The HT-2 mouse T cell line responds to both IL-2 and -4. We found that IL-4 initially antagonizes, and later synergizes with, IL-2-stimulated HT-2 cell proliferation in a dose-dependent manner. IL-4 also stimulates cell adhesion in static cultures. At the level of intracellular signaling, IL-4 antagonizes IL-2-stimulated activation of Akt, possibly through competition for limiting amounts of common receptor subunit and/or PI 3-kinase. Because IL-4 alone does not promote any Erk activation, we were surprised to find that IL-4 enhances IL-2-stimulated activation of Erk. IL-2/IL-4 co-stimulation provokes transient activation of STAT5 and prolonged activation of STAT6. This extended STAT6 activation may be critical in the IL-2/IL-4 induced synergy in T cell growth. Currently, we are investigating the crosstalk between these pathways and their functional roles in IL-2 and IL-4-stimulated T cell responses.

Intracellular Signaling Networks in the Immune Response: Pathways Activated by Interleukin-2 and Interleukin-4 Receptors and their Roles in T Cell Proliferation

by

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DEDICATION

This is dedicated to my two extraordinary nieces, Lydia and Celia, whose infinite energy and boundless curiosity always remind me to appreciate the small things in life.

BIOGRAPHY

Kristen Krupa Comfort was born on May 15, 1980 in Cleveland Heights, OH to Raymond and Wendy Krupa. As the youngest of three girls, most of her childhood was spent chasing after her older sisters, and striving to be like them. In 1998 Kristen graduated from Hudson High School (Hudson, OH) and decided to pursue a degree in engineering due to her love for science and math. She graduated with a B.S. of chemical engineering from the University of Dayton in 2002 and matriculated to North Carolina State University that fall to continue her education. September 4, 2004 she married her wonderful husband Donald Comfort, who recently completed his Ph.D. in chemical engineering from North Carolina State University. After successful completion of her M.S., Kristen will continue on for her Ph.D. in chemical engineering. Kristen, Don and their dog adorable dog Guinness will soon be relocating to Apex.

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INTRODUCTION AND OVERVIEW PART 1: IL-2 AND IL-4 INDUCED PROLIFERATION AND DIFFERENTIATION OF T CELLS AND THEIR FUNCTION IN THE IMMUNE RESPONSE

1.1 MOTIVATION AND RESEARCH SIGNIFICANCE

The ability of living cells to recognize and respond to chemical and physical stimuli in their surrounding environment is fundamental for life. The biochemical mechanisms responsible for processing this information inside the cell are collectively referred to as signal transduction. These mechanisms involve enzyme-catalyzed reactions and formation of noncovalent protein-protein and protein-lipid complexes (20). In the simplest case, an external perturbation binding to a surface receptor would initiate one signal cascade leading directly to the desired outcome. *(Figure 1.1)* However, most receptors are pleiotropic, meaning that they simultaneously trigger multiple signaling pathways and outcomes. Further, individual signaling pathways have the ability to modulate the activities of other pathways; through so-called crosstalk interactions. When also considering pathway cooperation and the presence of multiple concurrent stimuli, it becomes apparent that the dynamics of signal transduction are extremely complex (16, 27).

The cytokines interleukin-2 (IL-2) and interleukin-4 (IL-4) are key regulators of the immune system, particularly influencing the function of T lymphocytes. IL-2 induces a strong proliferative effect in T cells, whereas IL-4 causes differentiation (18, 22). When T cells are exposed to and other stimuli in concert, a synergistic effect transpires that generates rapid growth and differentiation, known as clonal expansion (43).

There is a strong desire to fully understand, at the molecular level, how this observed synergy arises, particularly the involvement of specific signaling proteins. Through quantification of the signal transduction network, it may be possible to fundamentally analyze and reproduce

cytokine-induced clonal expansion of T cells. This knowledge could be a powerful tool in bolstering the immune response in the fight against cancer and other diseases.

1.2 BACKGROUND

1.2.1 T cell function and its role in the immune system

The innate immune response provides the body's first line of defense against common microorganisms and is essential in combating bacterial infections. The cells of the innate system non-specifically target foreign pathogen for removal or destruction of infected cells. However, the innate immunity is not always sufficient to win this battle, and in some cases it fails to recognize its pathogenic opponents. Over time, the immune system has evolved to incorporate a more versatile means of guarding itself from these pathogens - the adaptive immune response. Adaptive immunity provides not only increased protection against a wide variety of foreign pathogens, but also a means of summoning this protection quickly in the face of a subsequent attack. The innate immune cells serve to activate and direct the cells of the adaptive immune system, showing how these two defensive fronts work together to fight off foreign invaders and infection.

The T lymphocyte, or T cell, is a key member of the adaptive immune system. T cells arise from the bone marrow, where they begin as hematopoietic stem cells. These stem cells have the ability to differentiate into one of many different immune cells including T cells, B cells, dendritic cells and several other leukocytes (45). These precursor T cells then leave the bone marrow and migrate to the cortex of the thymus, where they undergo a maturation process that involves gene rearrangement.

After successfully completing gene rearrangement, the cells begin the process of positive selection. An antigen presenting cell (APC) binds to and activates a T cell through its major histocompatibility complex (MHC), of which there are two classes: MHC class I and MHC class II. Immature T cells that recognize MHC class I develop into cytotoxic T cells (T_C cells), whereas cells that bind MHC class II mature into helper T cells (T_H cells) after further selection. Precursor cells that do not recognize either form of MHC do not receive a developmental signal from the APC and die of neglect (23, 52, 54).

Immature T cells then migrate into the medulla of the thymus and undergo the process of negative selection. The cells are presented "self" peptides by APCs and are tested to see if they respond. During this phase, any precursor cell that recognizes "self" is given a death signal that induces apoptosis and is thus removed from the repertoire (45, 52). Self-reactive T cells that mistakenly survive negative selection can lead to the development of several autoimmune diseases, causing the immune system to attack one's own body (19). This total process described above is referred to as clonal selection, which produces a collection of mature yet naïve T cells that each recognize a unique peptide sequence.

After a foreign pathogen enters the body, it is phagocytosed, broken down into small peptide sequences and displayed by an APC. Upon leaving the thymus each of these mature T cells, having now become a naïve T_C or T_H cell, circulates through the peripheral lymphoid organs and searches for the APC that is displaying the sole peptide sequence that it recognizes. Once a T cell finds its correct antigen, two independent signals must occur for it to transform from a naïve cell to an activated, armed T cell. First, the peptide/MHC complex must successfully bind to the T cell receptor and transmit a signal that the correct antigen has been encountered. A co-stimulatory signal must also be delivered to the T cell from the same APC for full activation and the induction of gene alteration (59). These co-stimulatory signals usually involve the interaction between molecules on the surfaces of the T cell and APC, such as CD28 and B7, respectively (13, 51).

After a T cell activated, it initiates the secretion of IL-2, which binds to the specialized IL-2 receptors on the T cell surface - driving cellular proliferation and differentiation (22). After rapid proliferation, cytotoxic T cells are ready to perform their effector function of seeking out and delivering lethal signals to infected cells in the body, whereas helper T cells must differentiate further.

Naïve T_H cells can differentiate into either T_{H1} or T_{H2} cells, which differ in the cytokines they produce as well as their functions. *(Figure 1.2)* T_{H1} cells activate macrophages, enabling

them to phagocyte and destroy pathogenic microorganisms more efficiently, whereas T_{H2} cells drive B cells to differentiate and initiate the production of antibodies. The cytokines secreted by a helper T cell subset establishes a positive feedback loop that promotes the differentiation of other T_H cells to that particular subset while simultaneously blocking the differentiation into the second subset (1, 38). For example, interferon-γ (IFN-γ) is produced in significant quantities from T_{H1} cells. IFN-γ can then favor the differentiation of T_H to T_{H1} cells while acting as an antagonist for T_{H2} development (6). On the other hand, the secretion of IL-4 from T_{H2} cells promotes the differentiation of more T_{H2} cells (18, 49). For the T_{H2} case, IL-4 can synergize with IL-2 with respect to growth, which greatly enhances T cell expansion and effectiveness (53).

After the adaptive immune system is activated, the foreign pathogen can be cleared by multiple routes. T_C cells, along with other specialized immune cells, recognize and kill tissue cells that have become infected by the pathogen. As a side note, the possibility of priming and activating T_C cells with IL-2 has been investigated as a mechanism for cancer therapy, however this treatment has been abandoned due to the fact that it is nearly impossible for the T_C cells to recognize and preferentially attack the tumor (21, 46).

At the same time, the macrophages activated by T_{H1} cells and the massive amounts of antibodies secreted by the B cells following activation by T_{H2} cells target and eliminate all the remaining pathogen. It becomes nearly impossible for a microorganism to survive such an attack, and following its removal, the immune system quickly returns the body to a state of homeostasis. After they have completed their function, most effector cells will die; however, a few T and B cells become memory cells, which continue to live and can readily proliferate upon reintroduction of the same pathogen.

1.2.2 IL-2, IL-4 and their receptors

IL-2 function

Interleukin-2 is a 15.5 kDa pleiotropic cytokine, secreted by activated T cells, that plays an immensely important role in activating the adaptive immune system; due to it's capability of serving as both an autocrine and a paracrine factor. In simple terms, a cytokine is a molecule secreted by one cell that acts upon another (40). Because IL-2 is critical for the clonal expansion of T cells, it is also commonly referred to as T cell growth factor (25). Clonal expansion consists of two concurrent processes: accelerated progress through the cell cycle and the induction of increased cell survival. In addition to its role in T cell growth, IL-2 mediates multiple biological processes, including growth and differentiation of B cells, creation of lymphokine-activated killer cells and expansion of natural killer cells (28). Recently, the role of IL-2 as a negative regulator of cell growth has also arisen. It is believed that after antigen eradication, IL-2 triggers activation-induced cell death (AICD), reversing its effect on T cell expansion and returning the body to homeostasis (9).

IL-2 receptor structure

After its secretion, IL-2 binds to a specific, multi-subunit receptor complex. The cell surface IL-2 receptor (IL-2R) is composed of three distinct subunits; the IL-2Rα, IL-2Rβ and γ_c (γ common) chains. *(Figure 1.3 a)* The IL-2Rα subunit is a 55 kDa membrane glycoprotein capable of binding IL-2. Although the α chain plays a critical role in ligand capture and stabilization of the complex, it does not directly contribute to intracellular signaling, due to an extremely short cytoplasmic tail (25). In contrast, the IL-2Rβ (p75) and γ_c (p64) chains together are both necessary and sufficient to initiate the signaling cascades.

The three subunits can be assembled to give various forms of the IL-2 receptor, which predictably display vastly different ligand affinities and signaling capabilities. Three distinct IL-2R complexes have been identified and are summarized in Table 1.1: a low affinity, an

intermediate affinity and a high affinity. Neither IL-2Rβ nor γ_c alone has significant affinity for IL-2 or is capable of inducing signal transduction (28). On the other hand, IL-2Rα by itself has a low but significant binding affinity for IL-2 (K_d = 10 nM). When IL-2Rβ and γ_c come together, they form an intermediate affinity receptor (K_d = 1 nM), which commences an low level of signaling (25). Finally, the trimeric IL-2Rα/IL-2Rβ/γ_c complex represents the "classic" high affinity IL-2 receptor (K_d = 10 pM) which signals efficiently after stimulation with biological concentrations of ligand (11, 25, 28).

A common theme in cytokine signaling is that the cytokines themselves are both pleiotropic and redundant. A possible explanation for this becomes clear when one considers the reoccurrence and sharing of receptor subunits among cytokines. For instance, the γ_c subunit utilized in the IL-2 system is also a component of the IL-4, IL-7, IL-9, IL-15 and IL-21 receptor complexes (40). Loss of γ_c can have wide-ranging consequences on the immune system, correlating with a cell's inability to respond to any of the aforementioned cytokines. This is demonstrated by the discovery that a deleterious mutation in γ_c causes severe combined immunodeficiency (SCID) in humans (37, 47).

It was quickly discovered that the majority of signaling events that occur after cytokine stimulation are mediated by the IL-2Rβ chain; hence, a functional analysis of this subunit was undertaken. Analysis of the amino acid sequence of the IL-2Rβ cytoplasmic region identified 3 distinct regions (15). The region closest to the plasma membrane was called the serine-rich region (S region) and consists of amino acids 267-322. Directly following the S region of the receptor was found to be an acidic-rich region (A region), comprised of amino acids 313-382. This is followed by the carboxyl terminal proline-rich domain (H region), which includes amino acids 378-525 (14, 15). IL-2Rβ mutants lacking one of these regions were created and served as the initial research tool in the determination of the functionality of each domain. For example, it became clear that S region was required for cell proliferation, and the A region was necessary for activation of tyrosine kinases such as Lck and Fyn (8, 26, 29). Further research into the sequence of IL-2Rβ revealed two highly conserved membrane-proximal sequences dubbed Box1 and Box 2, spanned by a variable domain termed the V

Box (39). Later, it became clear that characterizing the IL-2Rβ in terms of three domains was not specific enough, and a more detailed receptor analysis was undertaken.

IL-2 signaling initiation

The binding of IL-2 to its receptor complex induces a heterodimerization of the IL-2Rβ and γ_c cytoplasmic domains. Studies have shown that this heterodimerization is both required and sufficient for the initiation of IL-2 dependent intracellular signal transduction in T cells (34, 36). Many growth factor receptors have intrinsic tyrosine kinases, which upon ligand addition auto-activate and phosphorylate the receptor (50). This is not the case with IL-2 and most multi-subunit cytokine receptors.

After receptor dimerization, the tyrosine kinases Janus kinase 1 (Jak1) and Jak3 are recruited from the cytosol and non-covalently attach to the Box 1 and Box 2 motifs on the IL-2Rβ and γ_c, respectively (12, 24). Being in close proximity, the Jak kinases are able to sequentially transactivate each other via tyrosine phosphorylation (62). Once activated, it was found that the Jak kinases phosphorylate six specific tyrosine residues on the IL-2Rβ resulting in full activation of the receptor complex for signaling- four of which (Y-338, Y-355, Y-358 and Y-361) are located within the acidic-region and the additional two (Y-392 and Y-510) in the proline-rich region (9, 10). *(Figure 1.3 a)* Once phosphorylated, these specific, conserved tyrosine sites on the receptor subunit serve as docking sites for signaling proteins.

Other non-receptor tyrosine kinases, such as Syk, Lck and Fyn, bind to these phosphotyrosine sites and are poised to promote activate further signaling proteins that bind to IL-2Rβ (26, 30, 31, 56). There are three major cascades involved with IL-2 signaling that are responsible for the survival and proliferation of T cells: Jak/STAT, Ras/extracellular signal-regulated kinase (Erk) and phophatidylinositol 3-kinase (PI3K)/Akt (9); each of these pathways is discussed in greater detail in Chapter 2. In general, signals are transmitted through a cascade by phosphorylation or conformational changes, involving multiple signal

transducers and adapters, leading to either the amplification or attenuation of a cellular consequence (5).

IL-4 function

IL-4 is a multifunctional cytokine produced by activated T_{H2} cells, mast cells and basophils. In addition to its function in T cell differentiation, IL-4 plays several other decisive roles in the immune response. IL-4 can stimulate B cells to proliferate, induce their class-switching of immunoglobulin to IgE and IgG, and enhance their expression of class II MHC molecules (32, 33). During inflammation, IL-4 cooperates with tumor necrosis factor (TNF) to induce expression of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells, and it downregulates the expression of E-selectins. This shift in adhesion molecule expression by IL-4 is thought to favor the recruitment of T cells and eosinophils into an inflammation site and aid in healing (2). For millions of people, the overproduction of IL-4, especially in the respiratory system, leads to airway hyper-reactivity and the development of asthma. Asthma, generally triggered by allergic reactions, perfect illustrates why it is critical to maintain a proper balance of cytokines within the body (4, 58).

IL-4 receptor structure

The IL-4 receptor is a heterodimer composed of the 140 kDa IL-4Rα and the γ_c subunit. *(Figure 1.3 b)* The IL-4Rα binding epitope contains a series of acidic residues that attracts the large positively charged areas on the IL-4 surface created by several basic residues (32). This attraction is responsible for the extremely high affinity observed for IL-4Rα and its ligand (K_d = 20-100 pM) (35).

Over several years, four distinct domains within the IL-4Rα were discovered and characterized. The membrane proximal region contains the conserved "Box 1" and "Box 2" sequences, which serve as a docking site for Jak1. The second domain, the insulin/IL-4 receptor (I4R) domain, is critical for the initiation of proliferative and anti-apoptotic signals.

The gene-activating motif, the third region, is responsible for activating transcription factors that alter gene expression. The last section contains the immunoreceptor tyrosine-based inhibitory motif (ITIM), which is responsible for the downregulation of the proliferative signals induced by IL-4 (41).

As with the IL-2Rβ, it was determined that the IL-4Rα subunit contains several specific tyrosine residues that become phosphorylated and serve as docking sites for signaling proteins. The I4R domain contains one of the five tyrosine sites (Y-497), the gene-activating motif includes three conserved residues (Y-575, Y-603 and Y-631) and the remaining tyrosine site (Y-713) lies within the ITIM region (35). *(Figure 1.3 b)*

IL-4 signaling initiation

In a similar manner as IL-2, the binding of IL-4 causes the heterodimerization of its receptor subunits. The dimerization of the cytoplasmic regions is essential and sufficient for the initiation of IL-4 receptor signaling (55). Neither the IL-4Rα or the γ_c chain possesses endogenous kinase activity, therefore, the IL-4R requires receptor-associated kinases for the initiation of signal transduction. The non-receptor tyrosine kinases Jak1 and Jak3 bind to either the Box 1 or Box 2 motifs on the IL-4Rα and γ_c, respectively, followed by their transactivation. Very quickly after IL-4 engagement, Jak1 phosphorylates the five conserved tyrosine residues on the IL-4Rα, thus activating the receptor complex (44). Additional tyrosine kinases, Lck and Fyn for example, may also be recruited from the cytosol, bind to the IL-4Rα and help to activate other signaling proteins (55). IL-4 efficiently activates two major signaling cascades in T and B cells that lead to cellular proliferation; the Jak/STAT and PI3K/Akt pathways (17, 35). The IL-4Ris unique among cytokines and growth factors in that it does not incite the Ras/Erk signaling cascade, as will be further discussed in Chapter (42, 48, 60, 61).

IL-2 and IL-4 induced synergy

IL-2 and IL-4 are both T cell growth factors that employ many of the same mechanisms to accomplish their functions. When T cells are exposed to IL-2 and IL-4 simultaneously, they exert a synergistic effect that enhances the rapid expansion of the target cells (43, 57). Synergy must be accomplished by altering the balance of the intracellular signaling pathways involved in T cell proliferation and/or survival. At the same time, IL-2 and IL-4 receptors must compete for a common pool of γ_c subunits, suggesting an antagonistic relationship at high concentration levels. It is believed that this modification is in part due to the downregulation in functional IL-2R complex by IL-4, owing to the fact that both cytokines utilize the γ_c subunit (3, 7).

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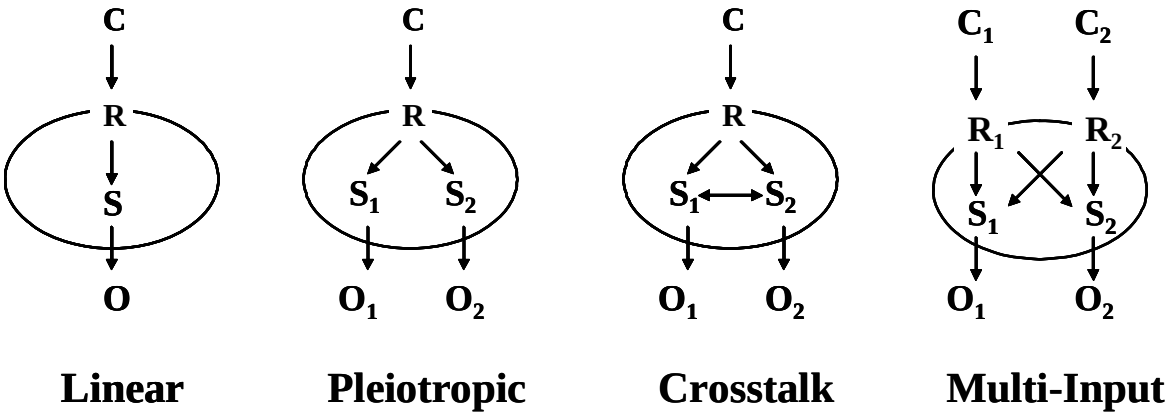
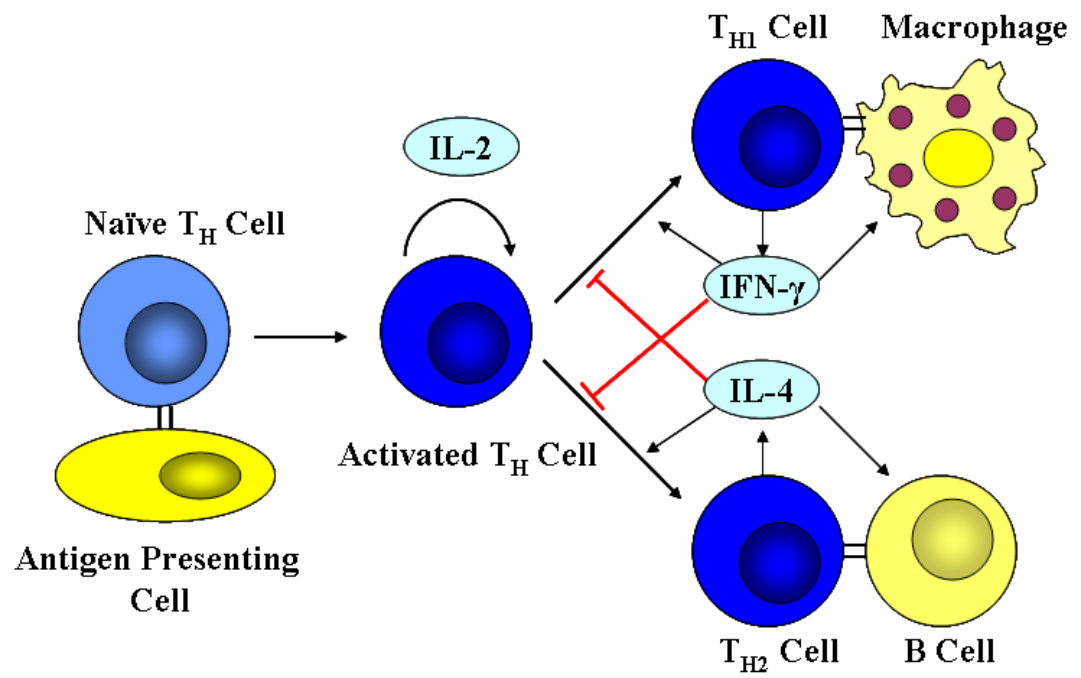
Table 1.1 IL-2 receptor compositions and ligand affinities

	Low	Intermediate	High
Subunits	α	β/γ_c	$\alpha/\beta/\gamma_c$
Approximate K_d	10 nM	1 nM	10 pM
Signaling?	No	Yes – Incomplete	Yes – Complete



Adapted from J.M. Haugh

Figure 1.1 Activation pathways of signal transduction networks Linear signal transduction networks are initiated by a perturbation, in this case a cytokine (C), binding to its specific receptor (R) and triggering the activation of a signaling pathway (S), which leads to a cellular outcome (O). A pleiotropic cytokine will give rise to multiple signaling pathways that may each control a unique outcome. As these multiple pathways have the potential to laterally influence each other, known as signal crosstalk, it adds another layer of complexity to the transduction network. Often more than one stimulus can concurrently affect a cell, with each growth factor activating several pathways that could potentially initiate crosstalk.



Adapted from J.M. Haugh

Figure 1.2 Cytokine induced T_H cell differentiation After a mature, yet naïve T_H cell encounters its specific antigen it secretes IL-2, inducing rapid proliferation. Once primed, a T_H cell can differentiate into either a T_{H1} or a T_{H2} subset, responsible for stimulating the development of either macrophages or B cells, respectively. This differentiation is cytokine driven, with IFN-γ provoking T_{H1} development and IL-4 stimulating T_{H2} cells. These cytokines not only trigger the differentiation of one T_H subset, but also act as an antagonist toward the other; for example IL-4 simultaneously induces T_{H2} progression while inhibiting T_{H1} differentiation.

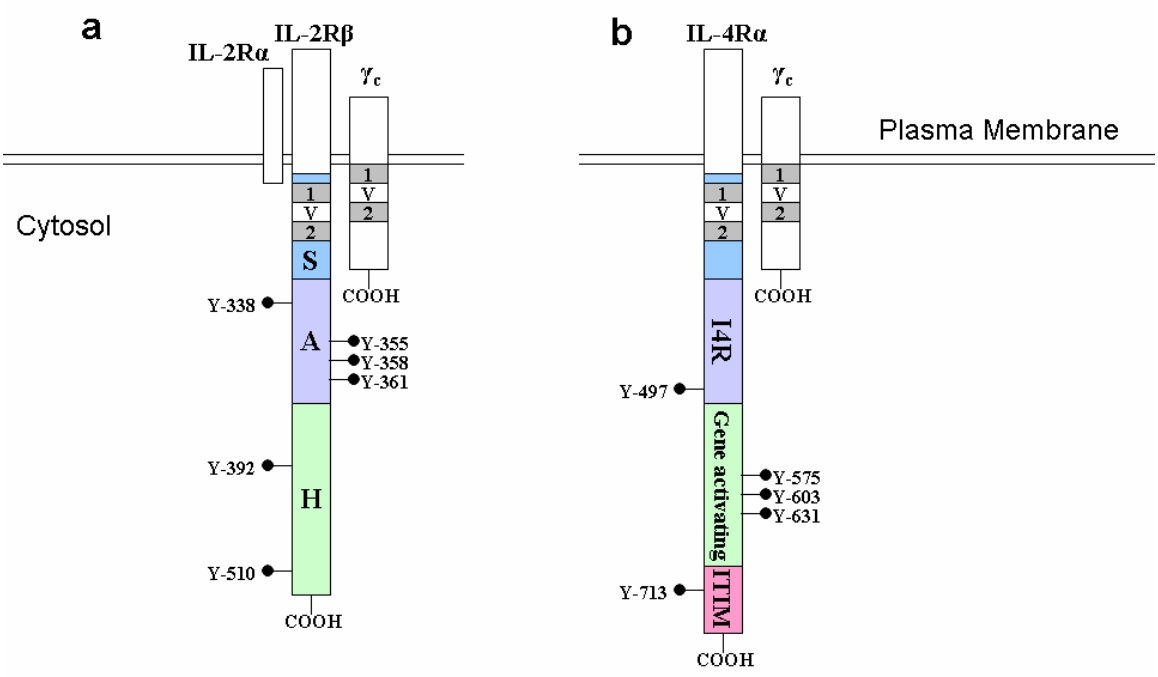


Figure 1.3 The IL-2 and IL-4 receptor complexes a) The IL-2R is composed of three subunits: the IL-2Rα, the IL-2Rβ and γ_c. Only the IL-2Rβ and the γ_c chains participate in signal transduction, with each containing a Box 1 and Box 2 motif to which Jaks bind. The IL-2Rβ can be functionally broken down into three domains: a serine-rich (S) domain, an acidic-rich (A) domain and a proline-rich (H) domain. Within the A and H domains lie six conserved tyrosine residues that serve as docking sites for signaling proteins following receptor activation. **b)** The IL-4R consists of the IL-4Rα and γ_c subunits, both of which contain the membrane proximal Box 1 and Box 2 regions necessary for receptor activation. In addition to the Jak binding region, the IL-4Rα contains three additional domains: the I4R region that binds with the IRS 1/2, the gene activating and the inhibitory ITIM domains. Within the IL-4Rα there exist five conserved tyrosine residues necessary for signal transduction.

CHAPTER 2

INTRODUCTION AND OVERVIEW PART 2: SIGNALING PATHWAYS STIMULATED BY IL-2 AND IL-4

2.1 BACKGROUND

Following IL-2 stimulation, three signaling pathways are induced: Jak/STAT5, PI3K/Akt and Ras/Erk. IL-4 stimulates the Jak/STAT6 and PI3K/Akt pathways, but is unique among cytokines and growth factors that it does not employ the Ras/Erk cascade. Chapter 2 will discuss each of these pathways in greater detail; including the members of each pathway, their protein structures, ways of activation, regulation mechanisms and downstream targets. Additionally, any known crosstalk mechanisms between these pathways will be divulged.

2.1.1 The Jak/STAT pathway

Overview

The Jak/STAT pathway is one of the most direct and simplistic cascades known to date in signal transduction. STATs (signal transducers and activators of transcription) are transcription factors that bind to the phosphotyrosine sites on a cytokine receptor, become activated via tyrosine phosphorylation by Jaks, dimerize and translocate to the nucleus, where they induce gene expression (31, 66, 67). STATs also are capable of triggering the activation of additional transcription factors, promoting progression through the cell cycle and initiating differentiation (112). The Jak/STAT pathway also induces several negative regulatory mechanism, without which the established signals would lead to uncontrolled growth and differentiation (104, 161).

The Jak family

Four mammalian protein tyrosine kinases have been identified in the Jak family: Jak1, Jak2, Jak3 and Tyk2 (66). Jak1, Jak2 and Tyk2 are widely expressed and are used in the activation

of several cytokine receptors, whereas Jak3 expression is highly regulated and binds solely to γ_c. Consequently, as seen with the γ_c subunit itself, inactivating mutations in Jak3 have been linked to the development of SCID, marked by a dramatic reduction in responsive lymphocytes (95, 134). Mutational studies in Jak family proteins have shown severe immunodeficiencies, non-responsiveness to cytokine stimulation and even death, making it clear that these four kinases possess non-redundant functions *in vivo* (80).

Analysis and comparison of Jak family sequences revealed seven regions of high homology, JH1-JH7. Although the biological importance of all these regions has not yet been fully elucidated, the function of several of the domains are well characterized. After noting that JH1 is critical for proper Jak activity, it was discovered to contain the kinase domain. Jak proteins are unique in containing two kinase domains, with JH2 represents a pseudokinase domain required for full catalytic activity (152). The JH3-JH4 regions share some similarity with Src Homology-2 (SH2) domains, but lacks phosphotyrosine binding ability. JH4-JH7 has been denoted the FERM (Four-point-one, Ezrin, Radixin, Moesin) domain and mediates the association of Jak with other proteins; JH7 binds specifically to the Box 1 motif in receptor subunits, while JH4-JH6 allows for associations with other Jak or non-receptor kinases (116, 152).

The role of Jaks in cytokine signaling

IL-2 and IL-4 receptors both lack intrinsic tyrosine kinases, so they must associate with non-receptor kinases to initiate signaling. Inactive Jak1 and Jak3 have been shown to quickly become coupled with IL-2Rβ or IL-4Rα and γ_c, respectively, following IL-2/IL-4 stimulation (116), although there is still some debate about whether these kinases are recruited from the cytosol or are pre-coupled to the receptor subunits in an inactive state. The JH7 domain of Jak proteins associates with the membrane proximal, proline rich conserved regions in the cytokine receptors, termed Box 1 or Box 2. This is the only known portion of the receptor where Jak1 and Jak3 will bind, and deletion of this region abrogates cytokine-induced signaling (66, 70, 106, 150).

Dimerization of the cytoplasmic domains of the receptor chains is required for signaling and is proposed to bring Jaks into sufficient proximity to allow for cross-phosphorylation and kinase activation. The Jaks in IL-2 and IL-4 induced signaling are turned on sequentially, with Jak3 activating Jak1 via tyrosine phosphorylation (175). Within the kinase domain of Jak1 and Jak3 there exists an activation loop that is regulated by two conserved tyrosine residues: Y1033 and Y1034 for Jak1 and Y975 and Y976 for Jak3 (91). In the case of Jak1, phosphorylation of Y1033 by Jak3 is required for kinase activation, while the second tyrosine is dispensable. Jak3 is unique in the respect that the activation-loop tyrosines are not absolutely required for catalytic activity. Even if Y975 and Y976 are not phosphorylated, Jak3 retains a low basal level of kinase ability, though it is greatly diminished compared to full Jak3 activation (91). This explains how Jak3 is capable of turning on Jak1 following cytokine stimulation, prior to phosphorylation of its own conserved tyrosine residues.

Following its activation, Jak1 is primarily responsible for priming the receptor complex by phosphorylating the conserved tyrosine residues, on IL-2Rβ or IL-4Rα (66). These phosphotyrosine residues can serve as docking sites for other protein tyrosine kinases, which bind to the receptor using their Src homology-2 (SH2) domains. Once a kinase is bound to the phosphotyrosine residues, Jak1 is already poised to turn on these proteins via phosphorylation (69, 175). These additional kinases play a crucial role in intracellular signaling by phosphorylating and activating other proteins that are bound to the receptor. Another responsibility of the Jak family is the phosphorylation of STAT transcription factors, after they bind to the receptor through their SH2 domains. STATs are the preferential substrate of Jaks, which will choose to phosphorylate them over other proteins (67, 116). The recruitment and full catalytic activity of Jak1 and Jak3 are essential for IL-2 and IL-4 induced signals; if either of these kinases is deleted or mutated, all of the ensuing signal transduction is abolished (106).

The STAT family

To date, the STAT family of transcription factors consists of seven members; STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (64). Each STAT protein has critical and non-redundant biological functions that operate through the cytokine receptor superfamily. Most of the STAT transcription factors mediate functions that influence the response of the immune system, underscoring the importance of these proteins not only for cytokine-stimulated signal transduction but for pathogen removal and maintenance of homeostasis (64).

Analysis of the STAT family has revealed six conserved functional domains. First, the 130 N-terminal amino acids represents a domain believed to regulate STAT nuclear translocation (80). The coiled-coil motif follows, consisting of four α-helices that when folded together form a large, predominantly hydrophilic surface that can interact with other helical proteins. Amino acids 320-480 form the DNA-binding domain (DBD), which contains the few residues capable of associating with DNA (116). Next is a small linker area that connects the DBD to the SH2 domain. The SH2 domain is an essential feature for protein activation and function, as it mediates both receptor and association and dimerization. The STAT family is the only group of transcription factors that requires a SH2 domain for operation (31). Finally, at the carboxyl terminus is the transcriptional activation domain (TAD) is located, which contains two conserved residues, one tyrosine and one serine, that must become phosphorylated for transcriptional activity of STAT proteins (31, 80).

Following cytokine stimulation and receptor activation, the STAT proteins are recruited to the receptor through their SH2 domains, whereupon they are tyrosine phosphorylated by the receptor-bound Jaks (116). Research has shown that Jaks must be present for phosphorylation of STATs to occur, suggesting that STAT proteins are a direct substrate of the Jaks (67). STATs may then dissociate from the receptor and dimerize through reciprocal phosphotyrosine/SH2 interactions. These dimers may then migrate to the nucleus, where they bind to DNA and induce target gene upregulation. Homodimers and heterodimers (in the

case of STAT5a and STAT5b), but not monomers, are capable of associating with DNA (31, 116). While phosphorylation of the tyrosine residue within the TAD is necessary for dimerization and translocation of STATs, it has been found that serine phosphorylation is required for maximal transcriptional activity. Serine phosphorylation of the STAT dimers is believed to stabilize the complex and prevent the proteins from uncoupling (163).

STAT5

It is believed that the STAT family of proteins originated from an initial STAT gene that underwent duplication multiple times. Nowhere is this more evident than when comparing STAT5a and STAT5b, which are over 96% homologous (111, 136). STAT5 was originally discovered as a transcription factor important in prolactin-dependent mammary gland development (64). It has since been determined that STAT5 plays an important role in the immune system, regulating B cell development, directing macrophage response to inflammation, and inducing T cell expansion (80, 116). Transcriptional activity of STAT5 is stimulated in T cells following treatment with IL-2 and has been proven to be critical for IL-2-stimulatedcell cycle progression and proliferation (153).

Following activation of the IL-2 receptor complex, STAT5 is recruited from the cytosol and binds to IL-2Rβ at one of two phosphotyrosine sites, Y-392 and Y-510 (45, 49). These two tyrosine residues are functionally redundant; only one site is required to elicit the full proliferative effect induced by STAT5 (43). Once bound to the receptor, Jak1 phosphorylates the conserved tyrosine residue located within the TAD sequence - Y-694 in STAT5a and Y-699 in STAT5b. After phosphorylation, the activated STAT proteins may dissociate from their docking sites, dimerize, translocate to the nucleus and bind to certain motifs in the DNA (89).

Once in the nucleus, STAT5 plays an important role in regulating transcription of target genes that are critical for T cell clonal expansion. One of the best characterized responses to STAT5 is the induction of the IL-2Rα gene. This leads to the increased presence of high

affinity IL-2 receptors, which helps promote the T cell response to IL-2 in an autocrine fashion (6). STAT5 also can control the transcription of several genes that are critical for cell cycle progression. Through knock-out studies, it has been shown that STAT5a/b controls the induction of cyclin D1, cyclin D2, cyclin D3 and cdk6, all of which have essential roles in cell cycle progression. Several proteins that are required for the proliferative response can also be induced by STAT5 binding, including *c-myc*, Bcl-x and Bcl-2 (92). STAT5 is also responsible for the upregulation of cytokine-inducible SH2-containing (CIS) proteins, which negatively regulate STAT activation and prevent excessive STAT-mediated signaling in T cells (89).

As with all intracellular signaling proteins that play a role in proliferation, lack of STAT5 negative regulation contributes to cancer development (2, 167). Several regulatory mechanisms are in place to help police the Jak/STAT5 pathway. Following ligand binding, the cytosolic protein-tyrosine phosphatase, SH2-containing protein 2 (SHP-2), is recruited to IL-2Rβ, where it is capable of dephosphorylating and deactivating both Jak1 and STAT5 (168). Further, as previously mentioned, STAT5 induces the expression of CIS-1, which negatively regulates Jak/STAT signaling by associating with Jak1 and blocking its catalytic activity. Additionally, CIS-1 can bind to the IL-2Rβ on the same phosphotyrosine sites as STAT5, reducing STAT5 activation by competitive inhibition (5, 101). A third mediator of STAT5 regulation is suppression of cytokine signaling 3 (SOCS-3), a member of a phosphatase family that regulates cytokine signaling. After IL-2 stimulation, SOCS-3 associates with the receptor, becomes activated by Jak1 and dephosphorylates receptor-bound STAT5, thus suppressing STAT5 induced gene expression (24). All of these cellular mechanisms in combination are capable of regulating STAT5 activation and cellular proliferation in response to IL-2, helping to maintain homeostasis and avoid tumorigenesis.

STAT6

STAT6 was the most recently discovered member of the STAT family of transcription factors (64). By knocking out its expression, it was revealed that STAT6 is required for T

cells to respond to IL-4 and differentiate into T_{H2} subsets and for B cells to engage in class switching and IgE production. STAT6-null mice also express a reduced pathology for asthma development and tumor formation, illuminating the role of IL-4 and STAT6 in these diseases (64, 80, 116).

Following IL-4 stimulation and receptor activation, STAT6 molecules translocate from the cytosol to the IL-4Rα, where they bind to the conserved phosphotyrosines Y-575, Y-603 and Y-631 (59, 159). After associating with the receptor, STAT6 monomers are tyrosine phosphorylated and activated by Jak1 on the conserved residue Y-641, after which they may dissociate from the receptor, dimerize, and migrate to the nucleus to activate transcription of target genes (165).

Activated, dimerized STAT6 is capable of both inducing the expression of and activating several other transcription factors to aid in signal transduction, including GATA-3, AP1, NFAT, NFκB and *c-maf* (52). The general transcription factor hierarchy in the IL-4 signaling cascade is as follows: STAT6 optimally induces the expression of GATA-3, which in turn induces *c-maf*, AP1, NFAT and NFκB in the nucleus. Once co-localized, this entourage of transcription factors selectively binds to target DNA segments and together initiate extensive gene expression (56, 57, 118).

The primary gene target of this collection of transcription factors is the IL-4 promoter, which contains binding sites for NFAT, NFκB, STAT6 and *c-maf*. Additionally, the IL-4 3' enhancer region specifically requires GATA-3 and NFAT binding to initiate transcription (87, 174). Beyond IL-4 ligand secretion, STAT6 also regulates the transcription of several other genes. A STAT6 DNA binding site has been identified within the promoter of the IL-4Rα gene, demonstrating how STAT6 contributes to IL-4 autocrine signaling (82). STAT6 also factors into IL-4-induced T cell cycle progression and is required for normal proliferation of lymphocytes. It has been shown that STAT6 inhibits the activity of p27kip, a protein that downregulates cdk2 and thus negatively regulates the G_1 to S phase transition (73).

As with all members of the STAT family, STAT6 must be carefully regulated to prevent excessive gene transcription. The c-terminus of the IL-4Rα contains an inhibitory ITIM motif, where SHP-1/2 may bind to Y-713 and dephosphorylate both the IL-4Rα and any local STAT6 (75, 79). As in IL-2 receptor signaling, the SOCS family plays an important role in inhibiting IL-4 signal transduction via STAT6. In the IL-4 system SOCS-1, and to a lesser extent SOCS-3, are capable of potently blocking the Jak1 and STAT6 response (93, 165). Finally, gene induction by STAT6 can be blocked by B cell lymphoma-6 (Bcl-6), a powerful transcriptional repressor that recognizes and associates with the same DNA elements as STAT6, competing for specific DNA binding sites. Interestingly, the transcription of Bcl-6 is initiated by STAT6 through a negative feedback loop; as STAT6 activity decreases, the expression of Bcl-6 correspondingly diminishes (34, 35). These mechanisms that control the signaling and gene induction following the activation of the Jak/STAT6 pathway are crucial in the regulation of T cell growth and differentiation.

2.1.2 The PI3K pathway

Overview

The phosphoinositide 3-kinases (PI3Ks) are a well characterized, conserved family of lipid kinases, which also possess a less understood serine-threonine protein kinase activity. A great variety of stimuli including, antigens, costimulatory molecules, growth factors and cytokines are all able to recruit and activate PI3K at the plasma membrane, upon which 3′ phosphoinositide (PI) lipids are produced (44). The lipid products of PI3K are important second messengers that bind and activate an array of cellular target proteins, which control cell proliferation, differentiation, survival, migration and metabolism (44, 117). As PI3K impacts numerous cellular responses, it is not surprising that activating mutations or overexpression of PI3K is linked with both cancer and leukemia (123, 156).

Structure and activation of PI3K

The PI3K family members are subdivided into four classes based upon their structural characteristics and substrate specificities: class Ia, Ib, II and III. Class Ia PI3Ks are activated in response to cytokine stimulation, and hence will be the only class discussed here (155). Class Ia PI3Ks are heterodimeric proteins comprised of a regulatory subunit and a catalytic subunit, which are constitutively associated.

There are five different isoforms of the regulatory subunit: p85α, p55α, p50α, p85β and p55γ; of these, p85α is the prototype. The p85α regulatory subunit consists of an N-terminal SH3 domain followed by a BcR homology (BH) domain that is flanked by proline rich sequences. The remaining structure of p85α is comprised of two SH2 domains, used to associate PI3K with tyrosine-phosphorylated proteins, separated by an "inter-SH2" domain that is responsible for binding to the catalytic subunit (155, 166). There are three genes for the p110 catalytic subunit of the type Ia class- p110α, p110β and p110δ. Structural analysis of the class Ia p110 catalytic domain revealed an N-terminal p85 binding domain, followed by a Ras binding domain, a C2 domain and the PI3K catalytic domain (76).

Prior to PI3K association with a phosphorylated protein, the p85 regulatory subunit inhibits the catalytic activity of p110. At the same time, p85 also stabilizes and prevents the degradation of p110 (169). The two SH2 domains of p85 can bind to certain phosphotyrosine sites, which induce a conformational change that allows the catalytic site of PI3K to be exposed. This change releases the inhibitory role p85 has over p110, and allows for full catalytic activity of PI3K (29, 68). p85 may also be tyrosine phosphorylated by Src family kinases, such as Lck or Fyn, on Y-688, which stabilizes this conformational changes and prolongs PI3K activation (158).

Following IL-2 stimulation, the IL-2R complex becomes phosphorylated and primed for induction of signaling cascades. Prior to receptor phosphorylation, p85 will not associate with the receptor in any way nor will PI3K become activated (103, 149). There are two

routes for PI3K activation in response to IL-2 stimulation, either through association with the collective receptor complex or through binding to Ras-GTP (68), the latter will be discussed in a later section. Because the IL-2R lacks direct binding sites for PI3K, how this cascade is activated remains somewhat controversial. It is established that p85 can associate with and be phosphorylated by tyrosine kinases that are bound to the IL-2Rβ. For example, both Lck and Fyn will preferentially bind to Y-355 or Y-361 on the IL-2Rβ and serve as a docking site for and activator of PI3K (54, 74, 145). The mechanism in which PI3K can be recruited and induced through a Shc/Grb2/Gab2 pathway is also well understood and documented (58). Shc is an adapter protein that binds to Y-338 of the IL-2Rβ and is capable of concurrently associating with other adapter proteins including Grb2 and Gab2 (Grb2 associated binding protein 2). The strong affinity of p85 for Gab2 tends to recruit PI3K to this complex where upon binding PI3K is activated (21, 58, 113).

How PI3K is activated following IL-4 stimulation is a well studied and understood process. Following receptor phosphorylation, the 170 kDa insulin receptor substrate-1 (IRS-1) binds to the phosphorylated Y-497 of the IL-4Rα and is phosphorylated by Jak1 (78, 160). IRS-1 has approximately 20 conserved tyrosine residues that can be phosphorylated by Jak1, making it capable of serving as a hub for several protein-protein interactions, including the recruitment of the PI3K regulatory subunit (143). Inactive PI3K translocates from the cytosol to the phosphorylated IRS-1 protein, where the p85 subunit binds via its dual SH2 domains, undergoing the necessary conformational change for PI3K activation (36, 170).

3′ phosphoinositide (PI) lipids

The major lipid substrate of active PI3K is phosphatidylinositol(4,5)biphosphate, PI(4,5)P_2. Class Ia PI3Ks phosphorylate this membrane lipid at the 3′ position of its inositol ring, producing phosphatidylinositol(3,4,5)triphosphate, also known as PI(3,4,5)P_3 or PIP_3 (85, 154). Although PI3K preferentially catalyzes the phosphorylation of PI(3,4)P_2, it will also convert PI(4)P to PI(3,4)P_2 or PIP_2. PIP_2 and PIP_3 are usually absent in quiescent cells, then appear within minutes of PI3K stimulation (61, 128). PIP_3 lipids act as second messengers

and recruit numerous signaling proteins that contain pleckstrin homology (PH) domains, due to the strong affinity of many PH domains for PIP_3 (124). Target proteins include Vav, TEC family kinases and p70 S6kinase, which are activated following IL-2 stimulation and help regulate proliferation (8, 108). However, the two principal effectors of PI3K signaling are Akt and 3-phosphoinositide-dependent protein kinase 1 (PDK1), which both have wide-ranging effects on cell behavior.

In order to maintain homeostasis, PI3K signaling must remain strictly regulated. In an effort to maintain this balance, several mechanisms are in place to control both PI3K and the lipid secondary messenger, PIP_3. The phosphatase SHP-2 controls PI3K-induced signaling by dephosphorylating Y-668 in the regulatory domain, increasing the likelihood that PI3K will dissociate from the receptor (29). Two proteins play an important role in the regulation of PIP_3 levels: PTEN (phosphates and tensin homologue deleted on chromosome ten) and SHIP-1 (SH2-domain containing inositol polyphosphatases-1) (7). PTEN was originally identified as a tumor suppressor, due to the fact that a deletion or mutation in PTEN can promote the development of cancer (37, 135). PTEN is a dual specificity phosphatase that can target either proteins or lipids, with its main substrate being 3′ phosphorylated phosphoinositides such as PIP_3. PTEN hydrolyzes PIP_3 to $PI(4,5)P_2$, specifically dephosphorylating the 3′ position of the inositol ring and reversing the actions of PI3K (96, 144). SHIP is a 145 kDa phosphatase that is activated by a wide variety of stimuli, including IL-2 and IL-4, and aids in controlling cellular survival and proliferation (98). SHIP-1 substrates include both signaling proteins and the lipid messenger PIP_3. In contrast to PTEN, SHIP removes the 5′ phosphate from the inositol ring of PIP_3 to generate $PI(3,4)P_2$, thus hindering PI3K dependent signaling (42, 131).

The role of PDK1 in Akt activation

Akt is a proto-oncogene and serine/threonine kinase that plays a central role in cell survival and proliferation. Akt is perhaps the most important, and definitely the most studied, downstream target of PI3K, because it influences the activation of numerous other signaling

proteins (11). The amino acid sequence of Akt reveals a very simple structure, consisting of an N-terminal PH domain, a catalytic region and a C-terminal regulatory domain (11).

The signaling pathway involved in Akt activation is fairly straightforward, though it contains multiple steps. Briefly, Akt binds to PIP_3 at the plasma membrane through its PH domain, causing a conformational change in the protein that relieves an autoinhibition of the active site (18). PIP_3 is also responsible for activating PDK1, another serine/threonine kinase. With these two kinases in close proximity, PDK1 threonine phosphorylates Akt on T-308, which lies in the activation loop (3, 13). Following phosphorylation of T-308, Akt is phosphorylated a second time on S-473 for full functionality (16). Determining which kinase is responsible for S-473 phosphorylation has proved to be a major challenge. It has been proposed that a second kinase, PDK2, exists to complete this task, though this protein has not been identified (3). It has also been suggested that PDK1 phosphorylates both Akt sites sequentially (147).

PDK1 was first discovered in the context of Akt signaling, however its importance in the activation of other signaling proteins has since been elucidated. PDK1 regulates the activity of several other protein kinases, including protein kinase A (PKA), protein kinase C(PKC), p90RSK and p70 S6kinase (83, 147). As a key regulator of these kinases and of Akt, with each exhibiting different cellular functions, PDK1 activation illustrates how PI3K can impact so many cellular processes.

Akt functions and targets

Through serine/threonine phosphorylation, Akt regulates several signaling proteins, all of which impact one of two cellular outcomes - cell survival or proliferation. While all three signaling pathways involved in the IL-2 response contribute to cell cycle progression and proliferation, the PI3K/Akt pathway is solely responsible for inhibiting apoptosis, or programmed cell death. First and foremost, Akt activates the B cell lymphoma 2 (Bcl-2) family, which is responsible for regulating apoptotic pathways. The two main pro-survival proteins, Bcl-2 and Bcl-x_L, prevent apoptosis by associating with the mitochondria and

maintaining the membrane integrity (26). If the protein cytochrome c is released from the mitochondrial membrane, it will activate Apaf-1, which then binds to and activates caspase-9, triggering apoptosis (26). Both Bcl-2 and Bcl-x_L are activated in an Akt-dependent manner and are necessary in preventing programmed cell death (1, 22, 54, 90).

Akt is also able to restrict pro-apoptotic members of the Bcl-2 family, such as Bax and Bad. Upon activation, Bax will associates with the mitochondria, where it binds to the membrane and advances the release of cytochrome c; however, once Akt phosphorylates Bax, it becomes sequestered in the cytosol (151). Bad encourages apoptosis by forming a heterodimer with Bcl-x_L or Bcl-2, preventing the pro-survival proteins from maintaining the stability of the mitochondrial membrane. After Akt phosphorylates Bad on S-136 and S-112, it becomes bound and sequestered by the adapter protein 14-3-3 (32, 33).

Additionally, Akt can directly phosphorylate caspase-9 on S-183 and S-196, blocking caspase-9 self cleavage, which in turn inhibits the caspase cascade and apoptosis (17). Similarly, it has been found that caspase-3 activity can be reduced by a PI3K-dependent mechanism, preventing DNA fragmentation (14). Lastly, how Akt dependent activation of NFκB leads to cell survival has been well studied. In the cytosol, NFκB is dimerized with a class of inhibitory proteins called IκBs, which prevents NFκB from migrating to the nucleus and initiating gene transcription. Following the activation of PI3K, IκB is phosphorylated by Akt, causing a conformational change that leads to the release of NFκB (72, 132). NFκB is then free to upregulate the expression of several anti-apoptotic proteins and assist in maintaining cell survival (41).

The second major role of Akt target proteins is to induce the cell cycle progression necessary to stimulate proliferation. Cell cycle progression is brought on by a two wave response consisting of an immediate burst of signaling followed by the activation of cyclin dependent kinases approximately twelve hours later (71). One downstream target of Akt that promotes cell cycle entry is E2F, a crucial regulator whose activation is required to pass the G_1 to S checkpoint (12). Secondly, the transcription factor *c-myc* is induced following Akt activation.

Although the exact mechanism behind the *c-myc* response is not understood, it is known that its expression enhances the progression through the cell cycle (1, 23). p27kip is a cell cycle inhibitor that binds to cyclin dependent kinases and blocks progress through the cell cycle. Akt opposes this interference by phosphorylating p27kip on threonine-157, causing it to be sequestered in the cytosol (25, 140). In a similar fashion, the obstruction of cell cycle progression brought on by the transcription factor FoxO is negated in a PI3K-dependent manner. After phosphorylation on T-24, S-253 and S-316 by Akt, nuclear translocation is halted, preventing the transcription of FoxO target genes (137, 142). While the number of downstream signaling proteins induced by PI3K/Akt are too numerous to discuss here, the aforementioned targets are some of the most critical in promoting survival and cell cycle progression.

2.1.3 The Ras/Erk cascade

Overview

Ras/Erk is the third major signaling pathway stimulated by IL-2, but, importantly, it is not activated in response to IL-4. Ras/Erk is also known as the mitogen-activated protein kinase pathway (MAPK) due to the fact that Erk (also known as MAPK) is the final kinase activated in the cascade. Ras belongs to a large superfamily of small GTPases that are critical for transmitting intracellular signals. Following IL-2R activation, Ras becomes activated, triggering a signaling cascade of protein kinases that include Raf, MEK and Erk. Activated Erk activates several transcription factors that influence a plethora of cellular fates (81). In response to IL-2, Ras/Erk regulates cell differentiation, proliferation, apoptosis and cell cycle progression in T cells (53, 55, 86). A significant percentage of human cancers are caused by activating mutations in the Ras/Erk pathway, leading to unregulated cell growth and differentiation (19, 102).

Ras: The GTPase family and activation

Ras is a member of the superfamily of small GTPases, whose activation states depend on the species of guanine nucleotide bound. Active GTPases are bound to guanosine triphosphate (GTP), whereas inactive forms are associated with guanosine diphosphate (GDP). In quiescent cells, most small GTPases are usually coupled with GDP, making them inactive. Following stimulation, the removal of GDP and the loading of GTP is facilitated by a class of enzymes known as guanine nucleotide exchange factors (GEFs). Since small GTPases have a low intrinsic GTPase activity, GTPase accelerating proteins (GAPs) are utilized in the hydrolysis of GTP to GDP, which completes the activation cycle and is crucial in the regulation of Ras and other GTPases (10, 100).

Four isoforms of Ras exist, H-Ras, N-Ras and two K-Ras proteins, which are nearly identical except for the hypervariable region at the C-terminus. This variability causes the Ras proteins to differ in their membrane targeting sequences, which affects their localization. H-Ras is the isoform most studied and is generally activated in response to growth factors or cytokines (139).

Stimulation of the Ras/Erk pathway by IL-2 begins with the recruitment and tyrosine phosphorylation of the adapter protein Shc, which binds to the IL-2Rβ solely at Y-338 through its phosphotyrosine binding (PTB) domain. Shc is a unique adapter protein in that it contains both a PTB and an SH2 domain, making it capable of coupling several proteins at once (40, 77, 125). Once Shc is bound to Y-338 on the IL-2R, it is phosphorylated on Y-239 and Y-317, priming it to serve as a docking protein for other signaling molecules (15, 171). Grb2 serves as a linker protein that binds the GEF *Son of Sevenless* (Sos) through one of its two SH3 domains. The Grb2/Sos dimer then couples with the phosphorylated, receptor-bound Shc bringing the exchange factor into close proximity with the membrane-bound Ras-GDP, promoting the formation of Ras-GTP (39, 50, 88, 119).

There is a possibility that another step is involved in the activation of Ras, though it remains controversial. This step involves the tyrosine phosphatase SHP-2, which may be required to achieve full activation of Ras (99). SHP-2 either binds directly to Shc or indirectly through the adapter protein Gab2 followed by its two-step activation. Once SHP-2 binds and both SH2 domains are engaged by phosphotyrosines, the two tail tyrosine residues, Y-542 and Y-580, become phosphorylated by neighboring kinases, activating the phosphatase (94). SHP-2 now can serve as a binding site specifically for the Grb2/Sos dimer, positively modulating the level of Ras activation. It has also been suggested that the phosphatase activity of SHP-2 is somehow involved in the recruitment and activation of Ras, though this mechanism has never been fully clarified (9, 114). Once GTP-bound, Ras is proficient in recruiting its target proteins to the membrane, which starts the signaling cascade.

Downstream signaling of Ras

The first target of Ras in the Ras/Erk pathway is the serine/threonine kinase Raf. Exactly how Raf is activated following its interaction with Ras-GTP is still under scrutiny (81). What is clear is that Raf activation involves membrane recruitment, the action of adapter proteins, conformational changes and phosphorylation (109). Once activated, Ras binds Raf kinase with high affinity. recruiting inactive Raf to the membrane. Raf binds to Ras-GTP through both a Ras binding domain (RBD) and a cysteine-rich domain (CRD) (97). If S-259 on Raf is phosphorylated, the adapter protein 14-3-3 can bind to that phosphoserine residue and inhibit successful Raf dimerization with Ras (109). The binding to Ras induces a conformational change in Raf, exposing conserved sites S-338 and Y-341 for phosphorylation by different kinases (97, 162). Following recruitment to the cell membrane, proper conformational changes and phosphorylation, Raf is eventually activated.

The principal downstream target of Raf is the dual specificity kinase MEK, also known as MAPK kinase. Within the catalytic domain of MEK lies two serine sites that need to be phosphorylated by Raf for the kinase to become functional: S-217 and S-221 (4). The inactivation of MEK, and the obstruction of the proliferative signal, requires the

dephosphorylation of both conserved serine residues (4, 27). MEK is responsible for the phosphorylation and activation the of serine/threonine kinase Erk. Erk is highly regulated and requires both tyrosine and threonine phosphorylation to be operative, specifically at sites T-183 and Y-185 (120, 173). Additionally, if either of theses sites becomes dephosporylated, Erk loses all of its catalytic activity (173). Following activation by MEK, Erk dimerizes and regulates several intracellular targets, notably certain transcription factors (121).

One target of activated Erk is the AP-1 (activating protein-1) family of transcription factors, which includes c-*jun* and c-*fos*. Erk regulates these proteins by serine phosphorylation, after which they form the homodimers or heterodimers necessary for DNA binding (20, 62, 107). Additionally, Erk activates Elk-1, another transcription factor that upregulates the expression of important signaling proteins such as c-*fos* (51). It has been proposed that in addition to its role in differentiation and cell cycle progression, c-*fos* also induces the expression of c-*myc*, creating a greater influence over cellular functions (105). However, the expression and activation of c-*myc* appears to be regulated by two pathways, one that is dependent and one that is independent of c-*fos* (23, 105).

Following the formation of Ras-GTP, a signaling cascade ensues resulting in the activation of scores of transcription factors (81). These transcription factors have the capability of activating and negatively regulating the expression levels of each other and themselves, helping to maintain a proper balance. These proteins have the power to control functions that are essential to T cell expansion, including IL-2 secretion, proliferation, survival and cell cycle progression (104, 126, 138).

Negative regulation of the Ras/Erk pathway

In order to prevent uncontrolled cell growth and differentiation due to Ras signaling, several negative regulatory mechanisms are in place. First and possibly most important is the involvement of Ras-GAP, which stimulates the GTPase activity of Ras. Ras-GAP directly binds to Ras, leading to hydrolysis of GTP to GDP and inactivation of Ras (146, 148, 157).

The phosphatase SHIP also plays an important role in the control of Ras signaling. It has been revealed that SHIP can bind directly to Shc following IL-2 stimulation, displacing the Grb2/Sos dimer (30, 98). Within the Ras/Erk pathway, there is also a negative feedback loop that controls signal output. Active Erk can phosphorylate Sos, causing it to dissociate from Grb2 (84). Lastly, there are several non-specific, cytosolic protein phosphatases that dephosphorylate the proteins involved in the Ras/Erk cascade. All of these mechanisms help control the cellular functions induced by Ras.

2.1.4 Signal crosstalk

No signaling pathway is able to elicit a full cellular response on its own. Distinct pathways often converge on select signaling molecules, which require multiple inputs for full activation and functionality. For example, both the PI3K and Ras/Erk pathways are needed to fully activate the anti-apoptotic protein Bad and the transcription factor c-*myc* (63, 127). This convergence is also seen at the level of the response brought on by these pathways. For example, full proliferation following IL-2 stimulation requires the activation of at least two of the three major signaling pathways involved (43, 110).

In addition to the convergence of two pathways on a single downstream target (e.g., transcription factor), the signaling proteins in one pathway have the potential to laterally modulate proteins in other pathways, altering the signaling dynamics and balance. One striking instance crosstalk in IL-2 signal transduction is the direct activation of PI3K by Ras-GTP (68, 76). The p110 subunit of PI3K can be directly bound and stimulated by Ras-GTP, activating PI3K (130, 141). The adapter protein Shc can also activate both Ras and PI3K simultaneously, with the aid of Grb2 and Gab2 (58, 60, 115). The co-localization of these pathways is the perfect opportunity for Ras to bind to p110 and positively modulate the PI3K pathway.

It has also been shown that PI3K can increase the activation of Ras, though the exact mechanism has yet to be clarified. Utilizing PI3K inhibitors, it has been shown that both

MEK and Erk are partially dependent on PI3K induction (28, 38, 164). One theory is that PI3K influences Ras through the PH domain-containing adapter protein Gab2. Following IL-2 stimulation, PI3K generates PIP_3 in the plasma membrane, which recruits Gab2 through its PH domain (48, 129). Grb2/Sos associates with the membrane bound Gab2 with surprisingly high affinity, allowing for Sos/Ras interaction to occur away from the IL-2 receptor complex (47, 172). This secondary mechanism is thought to increase signal transmission through the Ras/Erk pathway.

An additional instance of crosstalk between signaling pathways is serine phosphorylation of STAT dimmers by Erk. After tyrosine phosphorylation by Jaks, STAT molecules dimerize and translocate to the nucleus. During this process, or once within the nucleus, dimerized STATs are subject to dephosphorylation, causing them to disassemble and become inactive. Erk phosphorylation of STAT dimers stabilizes their association and helps protect against dephosphorylation from cytosolic or nuclear phosphatases (46, 65, 122).

The crosstalk that occurs between signaling pathways does not always enhance signaling; it can also serve a regulatory function. One well known example of this is the inhibition of Raf through Akt-mediated phosphorylation. Active Akt can phosphorylated Raf on S-259, which permits 14-3-3 binding to Raf, hindering its ability to associate with Ras and become activated (133, 176). The implication of this mechanism is that PI3K signaling can have either positive or negative effects on Ras/Erk pathway activation.

In reality, the signal transduction network is far more complex than depicted here; however, by focusing on the major pathways and key players involved, a general idea of what is occurring within the T cell following stimulation with IL-2 and IL-4 can be obtained. This approach allows several signaling molecules to be targeted for experimentation, in the hopes of quantifying not only the activation of these proteins but their interactions as well. The results of these studies may not completely divulge all the answers that were sought, but will hopefully direct future work; eventually lead to the determination of the key signaling proteins involved in IL-2 and IL-4 induced T cell expansion.

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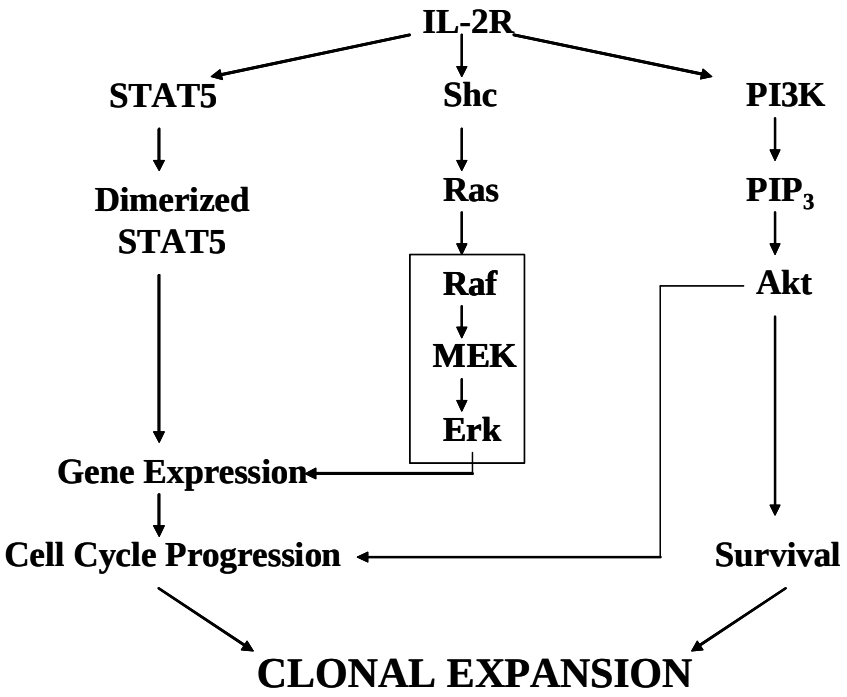
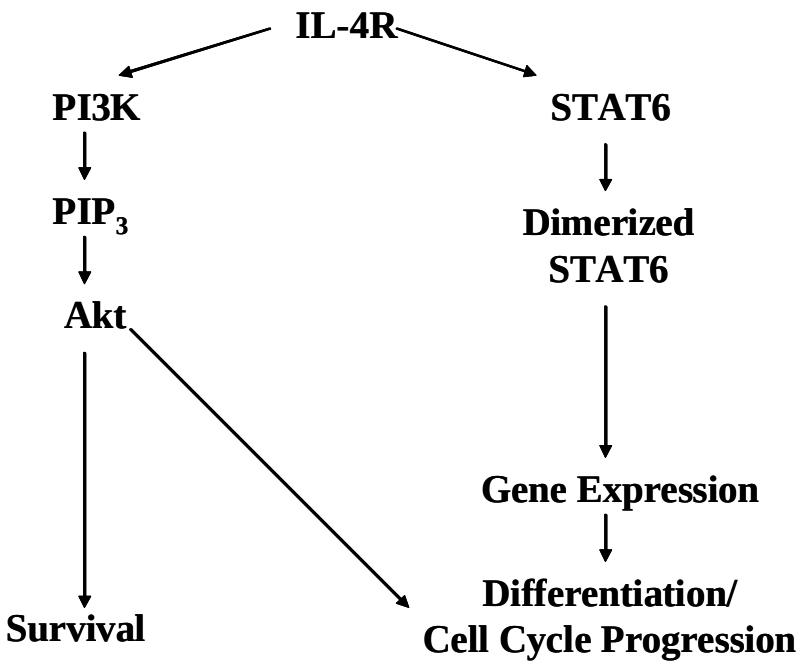


Adapted from J.M. Haugh

Figure 2.1 IL-2 induced signaling pathways Following IL-2/IL-2R ligation, three signaling cascades are activated – the Jak/STAT5, Ras/Erk and PI3K/Akt pathways. All three pathways cooperate to induce the cell cycle progression and survival necessary in to achieve rapid proliferation and differentiation, also known as clonal expansion.



Adapted from J.M. Haugh

Figure 2.2 The IL-4 dependent signaling pathways Following activation of the IL-4R, two major signaling pathways are activated – the Jak/STAT6 and PI3K/Akt pathways. This signal transduction network is essential for cell differentiation, cell cycle progression and survival. IL-4 is unique among cytokines in the respect that it does not employ the Ras/Erk pathway.

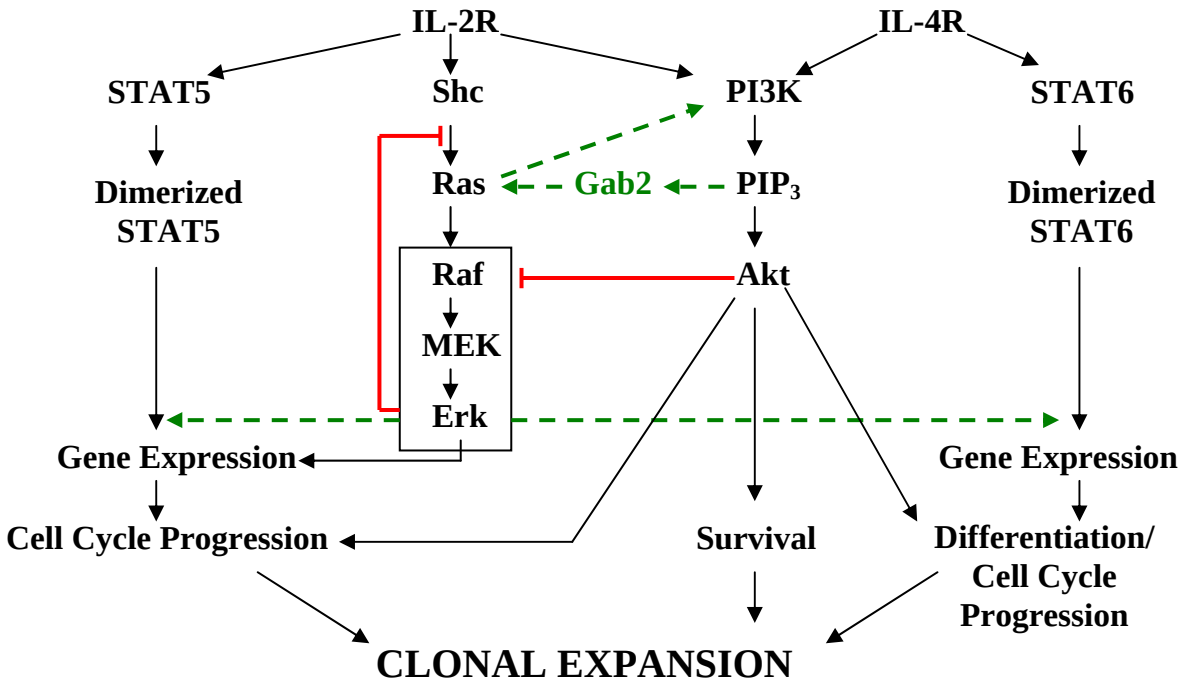


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Figure 2.3 Crosstalk in the IL-2/IL-4 system There are several possible crosstalk pathways occurring the IL-2/IL-4 co-stimulation system. Erk has the potential to serine phosphorylate the STAT dimers, increasing their stability. Ras is also able to positively modulate the activation of PI3K by activating the p110 catalytic domain. Following the creating of PIP_3 at the membrane by PI3K, Gab2 will be recruited and associate with Grb2/Sos, which in turn can activate Ras away from the receptor complex. It has been demonstrated that Akt may serine phosphorylate Raf, preventing its association with Ras. Additionally, Erk can phosphorylate and inhibit the exchange factor Sos, creating a negative feedback loop within the Ras cascade.

CHAPTER 3

THE T CELL PROLIFERATION RESPONSE TO INTERLEUKIN-2 AND INTERLEUKIN-4 AND THE SIGNALING MECHANISMS INVOLVED

3.1 INTRODUCTION

Cells sense and respond to chemical and physical stimuli through signal transduction pathways, which mediate cell proliferation, differentiation, migration, and survival. Signaling networks are the main process through which cells internalize biological cues and convert them to a cellular function (21). Interleukin-2 (IL-2) and interleukin-4 (IL-4) are key regulators of the adaptive immune response, mediating T cell clonal expansion and differentiation (20, 24). Additionally, the co-stimulation with these two cytokines leads to a synergistic effect on T cell growth, although the mechanism behind this effect has not been fully elucidated (42, 48). It is believed that the synergy induced by IL-2 and IL-4 is accomplished by altering the balance of intracellular signaling pathways induced in T cell proliferation.

The IL-2-dependent signaling events are induced by the binding of IL-2 to its high affinity receptor complex: composed of the IL-2Rα, IL-2Rβ and γ_c subunits (26, 34). In the case of IL-4, the ligand binds to a dimeric receptor, consisting the IL-4Rα and γ_c chains (37). Upon cytokine/receptor ligation, an intracellular dimerization occurs between the cytoplasmic tails of the receptor subunits, which triggers signaling initiation (36, 44). IL-2 receptors are known to activate the Ras/extracellular signal-regulated kinase (Erk), phosphoinositide 3-kinase (PI3K)/Akt and Janus kinase (Jak)/STAT5 pathways (11). IL-4 is unique among cytokines in that it does not activate Ras/Erk; it does, however, activate the PI3K/Akt pathway as well as a distinct Jak/STAT6 cascade (19, 37, 51).

Following IL-2 or IL-4 addition, Jaks 1 and 3 are the first signaling proteins recruited to the receptor complex, where they bind and phosphorylate conserved tyrosine residues on the IL-

2Rβ or the IL-4Rα (22, 25). STAT5 and STAT6 are the primary isoforms activated by IL-2 and IL-4, respectively, and following receptor association are targeted for phosphorylation by Jak1 (23). Dual tyrosine phosphorylation results in the STAT reciprocal dimerization, translocation to the nucleus and DNA binding (8).

The lipid and serine/threonine kinase PI3K, comprised of a p85 regulatory domain and a p110 catalytic domain, is activated following stimulation with either IL-2 or IL-4 and is responsible for cell proliferation and survival (10). After stimulation, PI3K is recruited to the receptor where upon binding to a Shc/Grb2/Gab2 trimer, the resulting conformational change induces activation (15, 33). Following activation, PI3K produces 3′ phosphoinositide (PI) lipids that act as second messengers, recruiting downstream signaling proteins to the plasma membrane (10). One central protein attracted to the plasma membrane is the serine/threonine kinase Akt, which becomes activated following association with the lipid messenger $PI(3,4,5)P_3$ and is responsible for the subsequent activation of numerous pro-survival and cell cycle progression proteins (1, 4).

The Ras/Erk cascade is a well characterized signaling pathway utilized by a variety of stimuli leading to cell proliferation and differentiation (14). Phosphorylation of the IL-2R complex leads to the formation of the Shc/Grb2/Sos trimer, which controls activation of the membrane bound Ras (32, 39). Ras then triggering a signaling cascade of protein kinases that include Raf, MEK and Erk, which are regulated by tyrosine, threonine and serine phosphorylation (3, 27, 40). Once active, Erk translocates to the nucleus where it phosphorylates additional transcription factors including *c-fos*, *c-jun*, Elk-1 and *c-myc* (7, 13, 41).

Although many studies have contributed to the knowledge of the roles of signaling proteins in T cell growth, very little has been done in the analysis of these proteins following co-stimulation with IL-2 and IL-4. Proliferation profiles under concurrent IL-2 and IL-4 stimulation revealed an initial antagonistic effect followed by synergistic growth in the murine T cell line HT-2. Additionally, both of these responses followed an IL-4 dose dependent fashion. The antagonism is thought to arise from a limitation of available γ_c

receptors, owing to the knowledge that both the IL-2R and the IL-4R require that particular subunit (6, 9).

We sought to determine, at the level of intracellular signaling pathways, what is responsible for this characteristic growth behavior following cytokine co-stimulation. Studies were performed that evaluated the activation level of several key signaling proteins as a function of time after IL-2 and IL-4 addition – mainly Akt, Erk, STAT5 and STAT6. An IL-4 dose dependent downregulation of activated Akt was observed shortly after cytokine stimulation; correlating with the antagonistic effect of IL-4 on cell growth. At extended time frames, the induced synergy was observed and was found to parallel high levels of active STAT6. Although it is doubtful that STAT6 alone is responsible for IL-2/IL-4 T cell synergy, it is clear that this transcription factor plays a key role in this behavior.

3.2 MATERIALS AND METHODS

Cell culture and reagents

The murine T helper cell line HT-2, obtained from American Type Culture Collection, is responsive to both IL-2 and IL-4 (17, 28). The culture medium consisted of Advanced RPMI 1640 base supplemented with 2% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol and 10 U/ml of recombinant human IL-2 (31, 50). The IL-2 was acquired from Peprotech, while the remaining cell culture reagents were purchased from Invitrogen. The HT-2 cells were subcultured in 75 cm^2 T flasks in an incubator at 37 ºC and 5% CO_2.

Recombinant murine IL-4 was bought from Peprotech. Antibodies, obtained from Cell Signaling Technology, include those against phospho-specific Akt (pSer 473), phospho-specific p44/p42 MAPK (pThr 202/pTyr 204), phospho-specific STAT5 (pTyr 694), STAT5, phospho-specific STAT6 (pTyr 641) and STAT6. Antibodies against Akt 1/2 and Erk-1 were from Santa Cruz Biotechnology. The anti-rabbit and anti-goat secondary antibodies were acquired from Cell Signaling Technology and Sigma, respectively.

Growth curve analysis

To determine HT-2 proliferation under specific conditions, growth analyses were performed in 48 well plates. Briefly, $3x10^4$ cells were seeded into each well, and the total volume was brought to 1 mL with media containing either 0 pM, 5 pM, 15 pM, 50 pM, 200 pM, 500 pM or 1 nM IL-2. Approximately every 12 hours, sampling occurred by mixing and counting the number of cells within a well using a Z_1 Coulter-Particle Counter (Beckman Coulter). The acquired data was used to construct growth curves that depicted the proliferative response to a certain media.

Preparation of detergent lysates

For short term stimulation experiments, HT-2 cells were cultured in a 1-L spin flask in cytokine-free media for 4 hours prior to stimulation to allow for signaling to return to a basal level. Cells were counted using a Z_1 Coulter-Particle Counter and inoculated at a density of $3x10^4$ cells/mL. After addition of cytokines, 25 mL of cell culture were removed at pre-determined time points, pelleted and resuspended in 200 μL of lysis buffer (50 mM HEPES pH 7.11, 100 mM NaCl, 1% Triton X-100, 50 mM β-glycerophosphate pH 7.3, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 5 mM sodium fluoride, 1 mM EGTA and 10 μg/mL each of aprotinin, leupeptin, pepstatin A and chymostatin) (16). All chemicals were obtained either from Fisher Scientific or Sigma. Briefly, cells were vortexed and incubated on ice for 20 minutes, then centrifuged at 6000 xg, at 4 ºC for 15 minutes to pellet the cellular debris. The supernatants were collected and the lysates were stored at –20 ºC. Cell samples were stimulated with the following IL-2/IL-4 conditions: 15 pM IL-2, 1 nM IL-2, 15 pM IL-2/10 pM IL-4 or 15 pM IL-2/300 pM IL-4.

For long-term stimulation, 30 mL of HT-2 cells were plated in a 15 cm non-treated petri dish at an initial density of $3x10^4$ cells/mL. When collecting samples, the plates were first scraped with cell lifters to remove any adherent cells from the surface, mixed and counted to determine cell density. Next, 25 mL of cells were pooled and lysed by the aforementioned method. The following stimulation conditions were used: 15 pM IL-2, 15 pM IL-2/10 pM IL-4 or 15 pM IL-2/300 pM IL-4.

Adhesion studies

To assess the effect of IL-4 on cell adhesion HT-2 cells were stimulated with varying cytokine concentrations. In non-treated 10 cm petri dishes, 10 mL of cells were plated at a density of $3x10^4$ cells/mL. At each time point, the numbers of cells in both the supernatant

and attached to plate were assessed using the Z_1 Coulter-Particle Counter. The cells were treated with 15 pM IL-2 with one of the following IL-4 concentrations: 0 pM, 1 pM, 10 pM, 100 pM or 300 pM.

Experiments were also performed to determine if, upon removal of IL-4, the cells would detach. The above experimental setup was repeated, and the cells were given four days to fully adhere. The media was switched and replaced with 15 pM IL-2 only, and an initial cell density on the plate was determined. Over several days, the numbers of cells in the supernatant and adhered to the plate were measured.

Quantitative Western blotting

Pooled cell lysates were subjected to SDS-PAGE to separate the protein contents. Samples were boiled for five minutes after addition of 4X Laemmli reducing sample buffer (Boston Bioproducts), loaded and separated in 10% acrylamide gels and transferred to PVDF membranes (29, 47). Membranes were blotted for proteins using the indicated primary antibodies and imaged using horseradish peroxidase-conjugated secondary antibodies and Super Signal West Femto Maximum Sensitivity Substrate (Pierce). The blots were imaged and quantified using a Bio-Rad Fluor-S MultiImager. Signaling protein activation was quantified by normalizing the amount of phosphorylated protein by the total amount of that protein, quantified in parallel.

3.3 RESULTS

3.3.1 IL-2 stimulates HT-2 cell proliferation in a dose-dependent manner.

The kinetics of HT-2 cell growth in the presence of varying IL-2 concentrations was also monitored *(Figure 3.1)*. Several important growth patterns were observed from these results. First, there is an initial lag in growth, approximately 48 hours long, which occurs while the cells are readjusting to their new environment. Next, an IL-2 dose-dependent growth phase arises, illustrating an IL-2 saturation for concentrations above 50 pM. This IL-2 dose-dependent growth pattern could be caused by a ligand depletion in the cultures with an initial IL-2 concentrations less than 50 pM. This possible ligand depletion effect is most significant after approximately 90 hours post stimulation, in static conditions. Focusing on the HT-2 growth following 15 pM IL-2 stimulation, it is observed that this concentration gives a definite response without saturating the IL-2 receptor complexes. All these results are consistent with a reported, effective K_d of approximately 10 pM (12, 26).

3.3.2 IL-4 antagonizes, but later cooperates with, IL-2-stimulated HT-2 cell proliferation in static culture.

At least in culture, both synergistic and antagonistic effects of IL-2 and IL-4 co-stimulation have been reported. Interestingly, we observed both behaviors in static culture *(Figure 3.2)*. IL-4 dose-dependent inhibition of IL-2 stimulated growth was seen at early times. Over time, concurrent stimulation with IL-2 and IL-4 clearly produces a synergistic response in T cells, again in a dose-dependent manner. Cells were incubated with either no IL-2 or a sub-saturating dose of IL-2 (15 pM) and various concentrations of IL-4 (0, 10 or 300 pM) IL-2 and IL-4 co-stimulation eventually yields a higher cell density than for either cytokine alone.

3.3.3 IL-4 stimulates adhesion of HT-2 cells in static culture.

IL-4 induces many significant changes in T cells, one of which is the expression of surface integrins and receptors which can mediates T cell adhesion and migration. Prior studies have demonstrated that following IL-4, but not IL-2, treatment, T cells are able to adhere to plastic dishes through an undetermined mechanism (38, 46). We therefore assessed the extent of HT-2 adhesion following IL-4 treatment *(Figure 3.3)*. It was found that the number of HT-2 cells adhered to a non-treated, polystyrene dishes increased in an IL-4 dose-dependent fashion with time *(Figure 3.3a)*. The addition of 1 pM IL-4 showed a negligible effect, whereas 100 pM and 300 pM IL-4 induced a dramatic adhesion response after 3 days in culture. After 5 days in culture, cells begin to detach from the plate, perhaps due to depletion of cytokines.

To further the adhesion response, we tested to see if continuous IL-4 stimulation was required to maintain HT-2 cell adhesion *(Figure 3.3b)*. Cells were pretreated under the same conditions used in the adhesion studies, allowing time cell attachment to occur. The IL-4-supplemented media was then removed and replaced with fresh media containing only IL-2. The numbers of adhered cells were monitored over several days; after approximately 3 days over half of the adhered cells had detached from the plates. This behavior was consistent in all the conditions that induced significant adhesion (IL-2 plus 10 pM, 100 pM or 300 pM IL-4). These results demonstrate that the IL-4-dependent HT-2 cell adhesion response is at least partially reversible.

3.3.4 Early signal transduction events induced by IL-2 and IL-4: IL-4 antagonizes IL-2 stimulation of Akt and STAT5 activation, but synergizes with IL-2 in the activation of Erk.

Cell lysates were collected over a span of eight hours in an attempt to characterize the initial stages of IL-2/IL-4 co-stimulation at the level of well-known signaling pathways. From the protein extracts, activation of the signaling molecules Akt, Erk, STAT5 and STAT6 were measured by quantitative Western blotting *(Figure 3.4)*. IL-4 antagonizes the activation of

Akt in response to IL-2 in a dose-dependent fashion, with significant inhibition seen in the presence of 300 pM IL-4 *(Figure 3.4a)*. Both IL-2 and IL-4 stimulate the PI3K/Akt pathway independently, but these results suggest that they do so to different extents; consistent with a receptor competition effect. Alternatively the inhibition of Akt might result from interactions between signals unique to IL-2 and Il-4 receptors.

Interestingly, the addition of IL-4 generated an augmentation of the IL-2 stimulated activation of Erk *(Figure 3.4b)*. This is notable because IL-4 alone does not activate the Ras/Erk pathway (results not shown). These results strongly suggest that there is significant crosstalk between IL-2 and IL-4 receptor signaling that converges on the Ras/Erk pathway. Finally, the STAT activation results are consistent with a receptor competition effect. IL-4 stimulated a significant decrease and increase in the activation of STAT5 and STAT6, respectively *(Figure 3.4 c&d)*. Even with equal IL-2 stimulation, levels of STAT5 phosphorylation were reduced in the presence of IL-4. A corresponding increase of STAT6 illustrates mounting signaling from the IL-4 receptor complex.

3.3.5 IL-4 induces prolonged activation of STAT6.

Extended time course were performed to further characterize IL-2- and IL-4-stimulated signal transduction in static cultures *(Figure 3.5)*. As in the shorter time courses, levels of activated Akt, Erk, STAT5 and STAT6 were quantified. Although the early activation responses of Akt, Erk and STAT5 are likely to functionally significant, all of these responses were downregulated to near basal levels within 48 hours (*Figure 3.5 a-c)*. In contrast, STAT6 activation remained high in an IL-4 dose-dependent manner *(Figure 3.5d)*.

3.4 DISCUSSION

In this study, we assessed the functional responses and signal transduction stimulated by IL-2, administered alone or in combination with IL-4. The mouse T cell line, HT-2, responded to both IL-2 and IL-4 as predicted, but the complexity of the response was somewhat surprising. IL-4 delays and then later enhances the rate of IL-2 stimulated HT-2 proliferation. Accordingly, IL-4 inhibits IL-2 stimulation of certain signaling intermediates while enhancing others. Although great headway was made, more work will need to be done to fully elucidate the mechanisms involved in the cellular response to concurrent IL-2 and IL-4 stimulation.

Potential role and mechanism of adhesion

IL-4 induced upregulation of the surface receptor protein CD8α on $CD4^+$ T cells (T_H cells) is well-documented, but has never been noted for IL-2 (18, 30). In addition, it has been found that following IL-4 removal, the induced CD8 expression diminishes (5). It is striking that this expression profile of IL-4 induced CD8 corresponds with and may be the cause of our observed adhesive response in HT-2 cells. It will be important to first confirm that CD8 is being induced by IL-4 in our cells and what signaling pathways are contributing to this response; STAT6-mediated transcription is a plausible mechanism for example. Another interesting study might be to determine what adhesive, if any, is present in the medium or secreted by the HT-2 cells. A likely candidate is an exopolysaccharide that is recognized by surface receptors (43). The adhesive response may also be due to the presence of a yet unknown receptor or ligand induced by either IL-4 or IL-4 in combination with serum. IL-4 induced expression of numerous integrins on the T cell surface, including E-Selectin, L-Selectin and Very Late Activating Antigen 4 (VLA-4), may also be the cause of HT-2 adhesion to plastic dishes (35, 45). It would also be advantageous to establish the role that cell adhesion plays in IL-4 induced proliferation. If the surface receptor expression were determined and could be reduced by siRNA, or other methods, it would be interesting to see

if the HT-2 cells still adhere in response to IL-4 and if the synergistic growth in response to IL-2/IL-4 co-stimulation was affected.

IL-4 antagonism of IL-2-stimulated signaling is consistent with a competition mechanism

It has been supposed that the antagonistic effect of IL-4 on IL-2-stimulated growth, when observed, is caused by competition for limiting amounts of the γ_c receptor subunit. The short-term analysis of Akt activation showed a decreased in signaling with IL-4 addition, even though both IL-2 and IL-4 activate the PI3K/Akt pathway. If this is caused by competition for γc or other common components, it must be assumed that the IL-4R is not as efficient as the IL-2R in the activation of PI3K/Akt. This is currently being tested by comparing Akt phosphorylation in cells stimulated with IL-2 or IL-4 alone, or co-stimulated with IL-2 and IL-4. Further support for the competition mechanism is found in the inhibition of IL-2 stimulated STAT5 activation by IL-4. If this mechanism is correct, the effect of IL-4 on Akt and STAT5 activation should disappear for saturating conditions of IL-2 (e.g., 1 nM). We are currently performing these experiments.

The mechanism and functional importance of signaling crosstalk

Our results also indicate that there is significant crosstalk between the IL-2R and the IL-4R at the level of Erk activation, thought the underlying mechanism remains to be elucidated. Utilizing pharmacological inhibitors may help to clarify this issue. For example, if blocking PI3K activity with drug LY 294002 (49) results in a change in the Erk response, it would establish an interaction between these pathways. Additional studies with LY 294002 could help strengthen the argument that a decrease in Akt activity is inducing the inhibition of IL-2 stimulated cell growth by IL-4 at early times.

Similarly, blocking MEK, and thus Erk, activation with PD 098059 would resolve the question of how strongly Erk influences the other signaling pathways (2). Whether Erk is capable of stabilizing the STAT6 dimer is still unknown and could possibly be determined by

analyzing lysates obtained following co-stimulation with IL-2/IL-4 in the presence of PD 098059. We also plan to us the MEK inhibitor to ascertain the role of Erk in the IL-2/IL-4 stimulated HT-2 cell proliferation.

Synergistic growth and the possible role of STAT6

Studies have revealed the existence of a synergistic effect on T cell growth following the co-stimulation with IL-2 and IL-4. During this time course, it was found that the activity of STAT6 was elevated, whereas the levels of active Akt, Erk and STAT5 were downregulated. It is therefore tempting to speculate that STAT6 plays a central role in the induction of IL-2/IL-4 growth synergy. This supposition is consistent with the action of STAT6 in inducing other transcription factors and also IL-4 secretion, potentially establishing an autocrine STAT6 activation loop. Although STAT6 activation is, in all probability, not the only necessary trigger for synergy, it can serve as a foundation for future work. One study that would shed more light on the role of STAT6 is to knock down its expression, e.g. using the siRNA approach. After removing the effects of STAT6 and repeating the growth analysis, it could be determined if STAT6 is required for IL-4R-mediated inhibition or enhancement of IL-2-stimulated growth at early and later times, respectively. Looking beyond STAT6, other signaling proteins could be targeted for future study as well. The most promising candidates would be transcription factors that STAT6 activates, including GATA-3, NFAT and NFκB.

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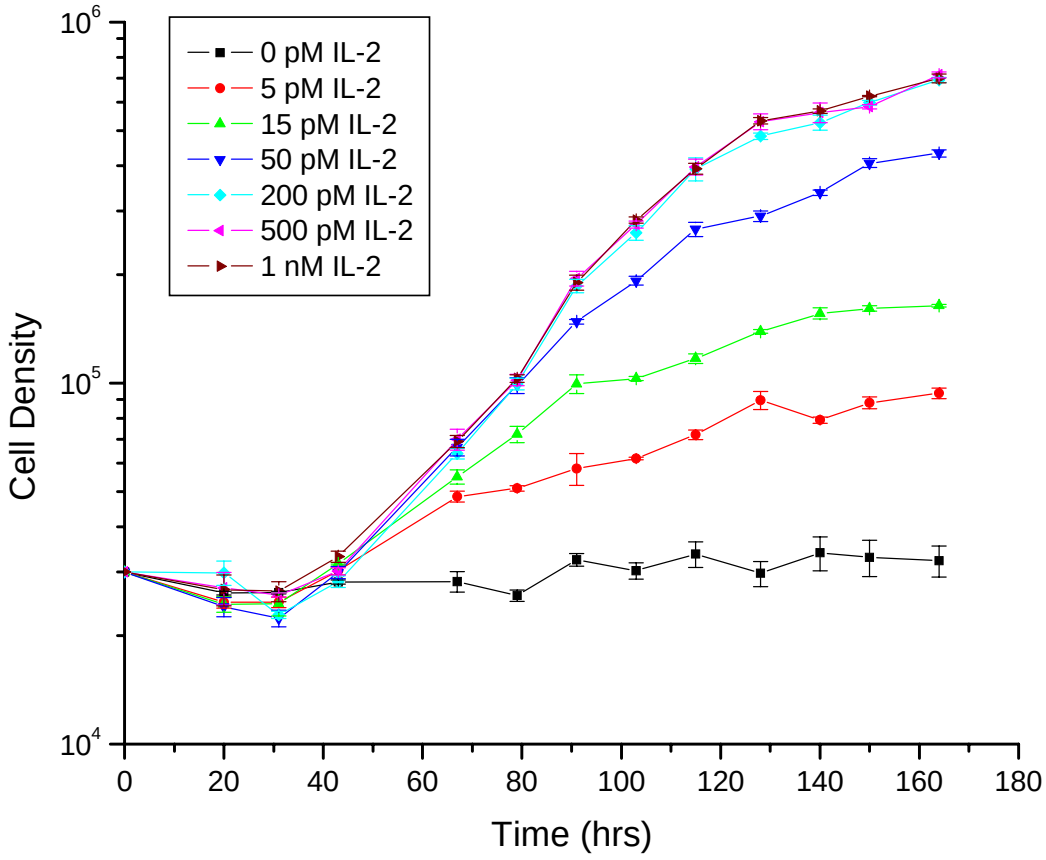

Figure 3.1 IL-2 dependent growth of T cells HT-2 cells were incubated with varying IL-2 concentrations to characterize the proliferation response of the cell line. (mean ± S.E., n=3)

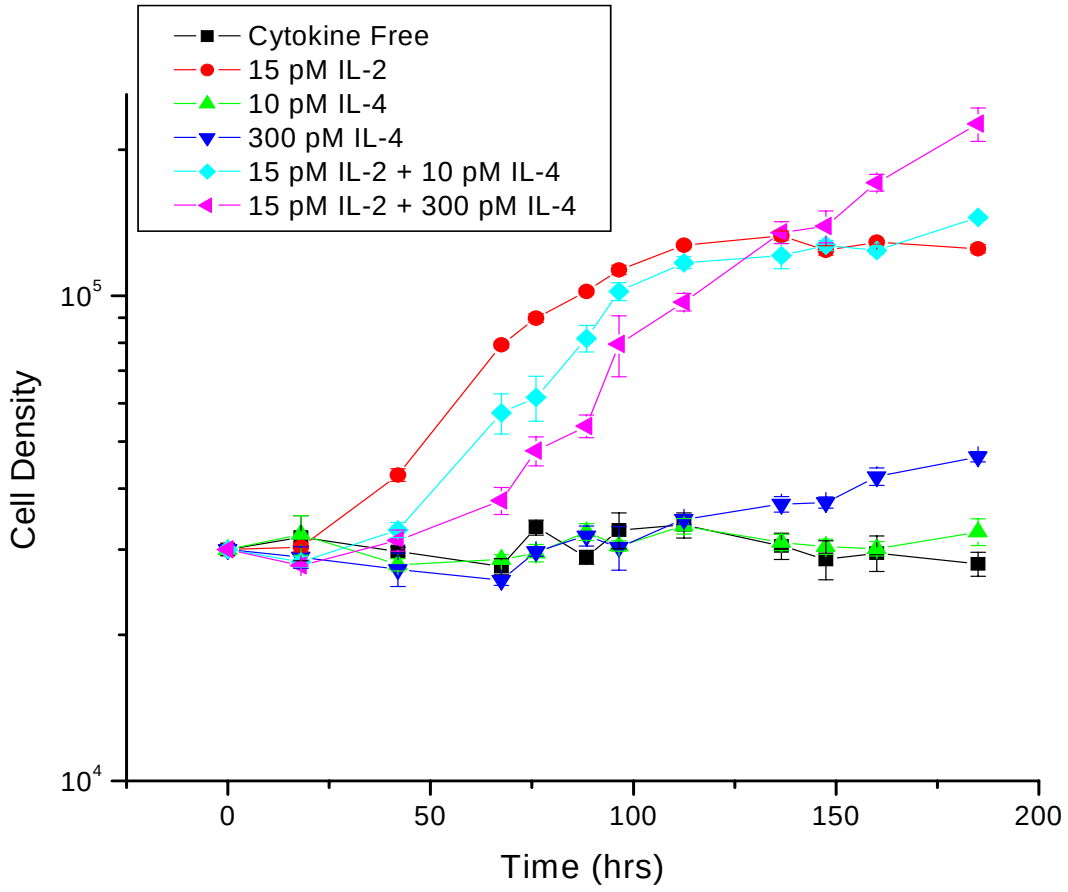

Figure 3.2 Growth response to IL-2 and IL-4 co-stimulation Growth kinetics of HT-2 cells were monitored following stimulation with IL-2 only, IL-4 only and IL-2 or IL-4 together. (mean ± S.E., n=3)

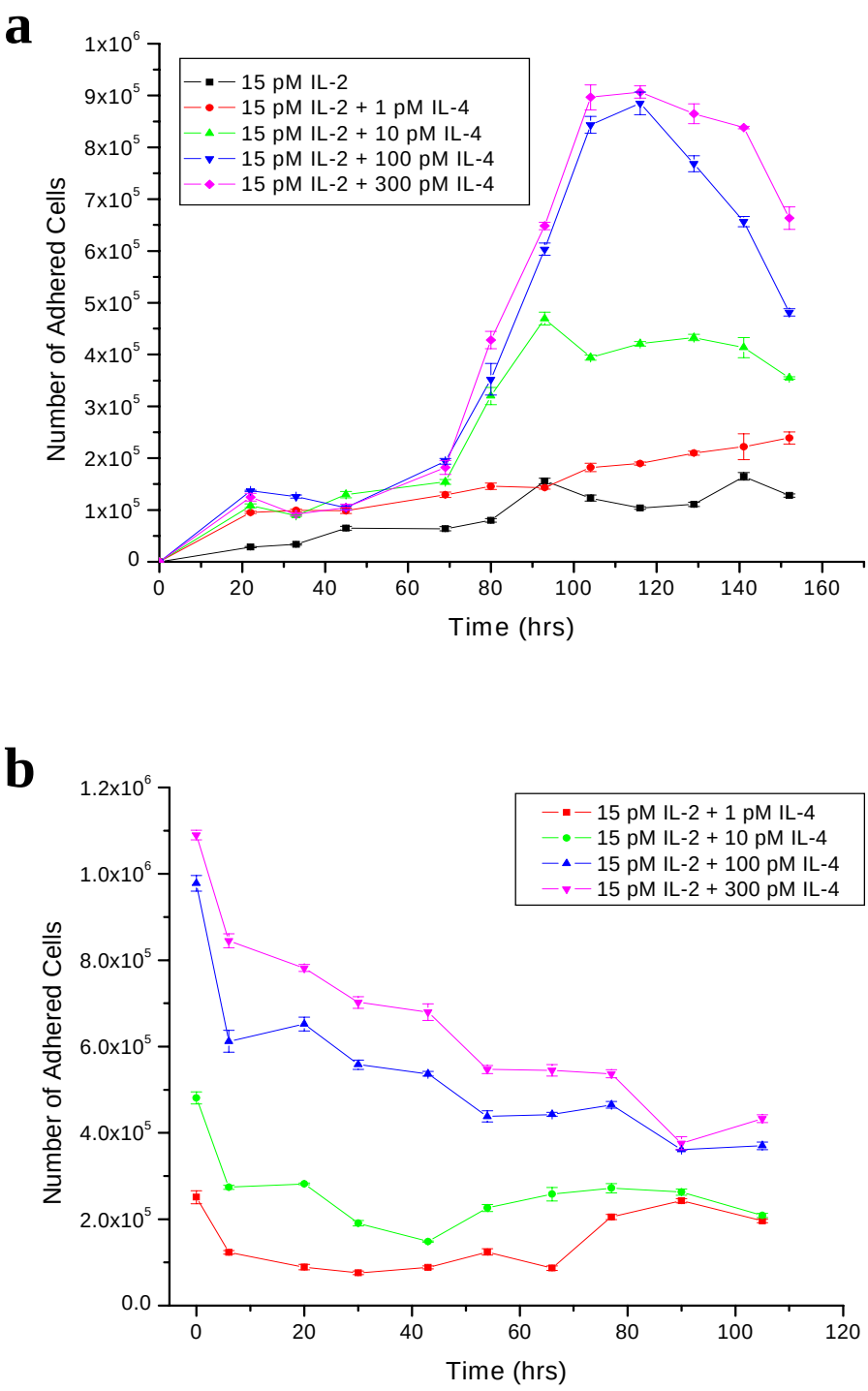

Figure 3.3 IL-4-induced adhesion of HT-2 cells a) Following IL-4 addition the number of cells that adhered to a 10 cm non-treated petri dish was monitored over time. The HT-2 cells attached to the plate after IL-4 induced expression of a yet unknown surface receptor. **b)** Cells were allowed to adhere for 4 days in the presence of the cytokines indicated, after which they were incubated with 15 pM IL-2 only for the times indicated. (mean ± S.E., n=3)

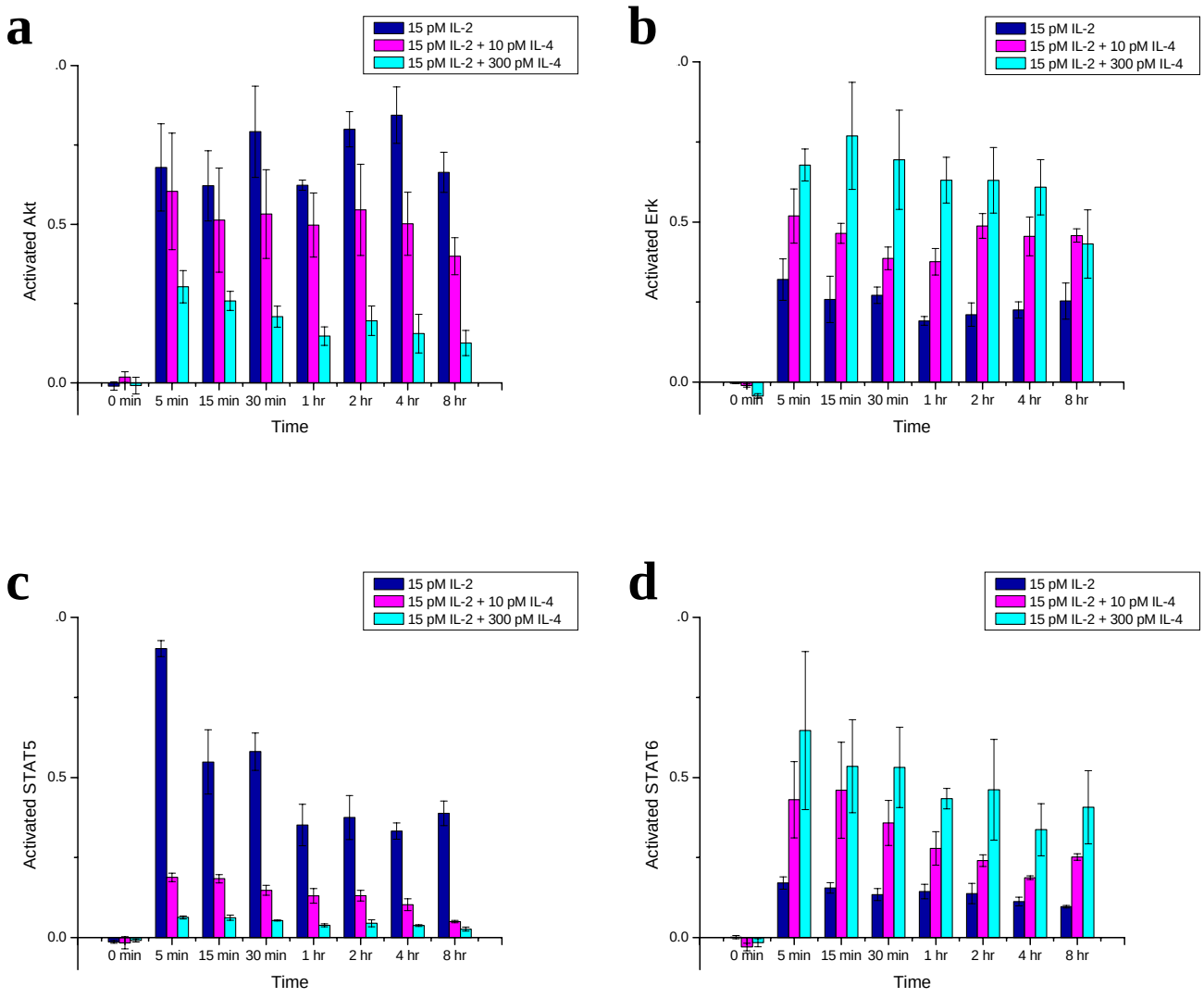

Figure 3.4 Early signal transduction responses to IL-2 and IL-4 co-stimulation HT-2 cells were cultured with a 1-L spin flask in either IL-2 only or IL-2 and IL-4 containing medium. Over a period of eight hours, samples were collected and lysed. Quantitative Western blotting was performed as described in Section 3.2. **a)** Akt phosphorylation **b)** Erk phosphorylation **c)** STAT5 phosphorylation **d)** STAT6 phosphorylation (mean ± S.E., n=3)

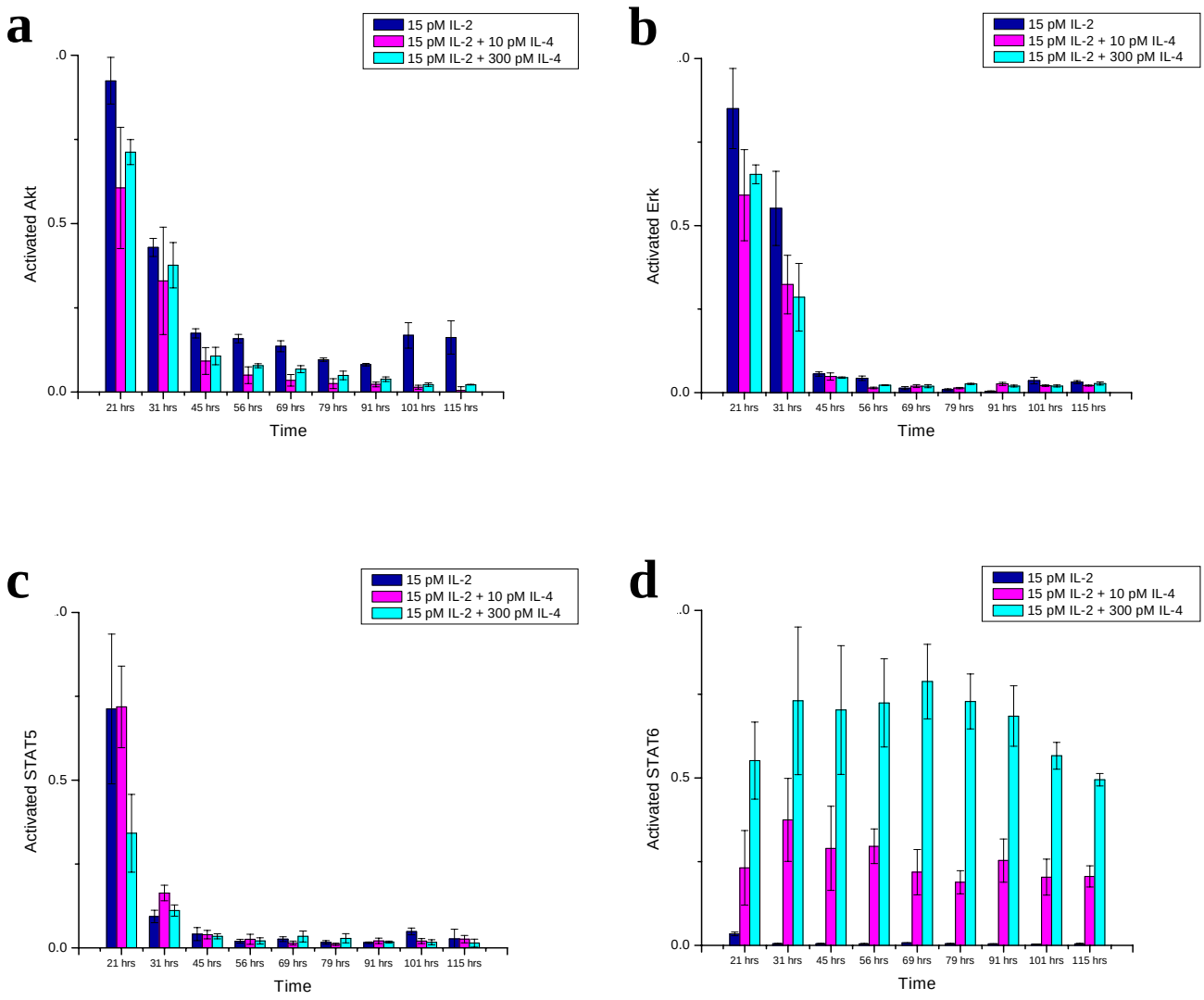

Figure 3.5 Long-term signal transduction responses to IL-2 and IL-4 co-stimulation Cells were stimulated with either IL-2 or IL-2 and IL-4 and cultured statically in 15 cm non-treated petri dishes. Quantitative Western blotting was performed as described in Section 3.2. **a)** Akt phosphorylation **b)** Erk phosphorylation **c)** STAT5 phosphorylation **d)** STAT6 phosphorylation (mean ± S.E., n=3)