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Signaling Pathways Activated by Interleukin-2 and Interluekin-4 Receptors Mediate T Lymphocyte Clonal Expansion

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ABSTRACT

COMFORT, KRISTEN KRUPA. Signaling Pathways Activated by Interleukin-2 and Interluekin-4 Receptors Mediate T Lymphocyte Clonal Expansion. (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 IL-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/Akt 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 IL-4. We found that IL-4 initially antagonizes, and later synergizes with, IL-2-stimulated HT-2 cell proliferation in a dose-dependent manner. Additionally, transient signaling through the PI3K/Akt and Ras/Erk pathways are required for optimal T cell proliferation. IL-4 stimulates cell adhesion in static cultures, again in a dose-dependent fashion, and it partially inhibits IL-2-stimulated activation of Akt, Erk and STAT5, consistent with a competition effect. IL-2/IL-4 co-stimulation provokes transient activation of Akt, Erk and STAT5 coupled with prolonged activation of STAT6. This signaling profile and the cell adhesion response suggest potential mechanisms by which IL-4 influences the proliferation of helper T cells.

Signaling Pathways Activated by Interleukin-2 and Interluekin-4 Receptors Mediate T Lymphocyte Clonal Expansion

by

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DEDICATION

This work is dedicated to two of the most important and influential people in my life – my sisters. Kari and Kelly, your unwavering love and encouragement has supported and sustained me during the tribulations as well as the successes.

I also dedicate this document in memory of Brooke Bossman. Even though we were born cousins, you were always so much more to me: you were my third sister. Only through the pain of losing you was I able to discover my true inner strength.

BIOGRAPHY

Kristen Krupa Comfort was born on May 15, 1980 in Middleburg 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. in chemical engineering from the University of Dayton in 2002 and matriculated to North Carolina State University to continue her education. On September 4, 2004 she married her wonderful husband Donald Comfort, who also obtained his Ph.D. in chemical engineering from NCSU. Kristen, Don and their adorable dog Guinness currently live in Apex, NC. Kristen and Don are expecting their first child, a baby girl, in December 2007. Kristen is currently looking for employment with a biotechnology company in the Raleigh area, however, first plans to take a few months off to give birth and spend time with her baby.

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My experience here at North Carolina State University has been memorable and wonderful, mostly due the people I have met along the way. First and foremost, I would like to thank my advisor, Dr. Jason Haugh, for his guidance, support and patience with regards to both myself and to this project. The Haugh group members, past and present, have been invaluable in their encouragement, ideas and friendship: especially Ian Schneider, Michael Weiger and Adam Melvin, who made work truly enjoyable. I would also like to thank the members of the Kelly laboratory, specifically Shannon Conners, Josh Michel, Jason Nichols, Amy VanFossen and Kate Auernik, who always treated me as member of their own group. Additionally, I would like to acknowledge Sandra Bailey, the graduate secretary, whom I believe is truly responsible for holding this department together.

I owe a special thank you to all my friends, both from UD and NCSU, whose confidence, loyalty and enthusiam sustained me throughout the past few years. To all my family for their unconditional love, especially my sisters who have stood by me my entire life and were always there when I needed them. I am grateful for the freedom my parents always gave me, allowing me to follow my dreams, no matter where they took me. For my daughter: knowing that you were on the way gave me the strength to continue on when I didn't have one ounce of energy left in me – which was also due to you. To my wonderful husband Don, thank you for your never ending love and support: without you behind me I wouldn't be where I am today.

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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 (24). In the most simplistic scenario, an external perturbation binding to a surface receptor would initiate one signal cascade leading directly to a cellular 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 (20, 31).

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 (22, 26). When T cells are exposed to these stimuli in concert, a synergistic effect transpires that generates rapid growth and differentiation, known as clonal expansion (48).

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 experimental 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.

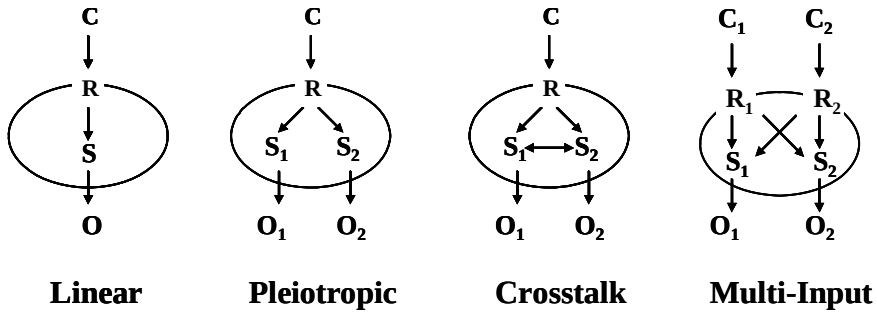

Figure 1.1 Activation pathways of signal transduction networks Linear signal transduction networks are initiated by a perturbation, in this case a cytokine (C), binding to its specific receptor (R) and triggering the activation of a signaling pathway (S), which leads to a cellular outcome (O). A pleiotropic 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 affect a cell, with each growth factor activating several pathways that could potentially initiate crosstalk: creating a very convoluted system.

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 even recognize its pathogenic opponents (8). 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 (62). 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 (12). 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 (50). 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 (2).

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) following further selection. Precursor cells that fail to recognize either form of MHC do not receive a developmental signal from the APC and die of neglect (27, 57, 59).

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 (50, 57). 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 (23). The above process, in its entirety, is referred to as clonal selection and results in the production of a mature yet naïve T cell population that each recognizes 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 (46). 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 (65). 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 (17, 56).

After a T cell becomes 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 (26). 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 undergo further differentiation.

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 phagocytose and destroy pathogenic microorganisms more efficiently, whereas T_{H2} cells drive B lymphocytes to differentiate and initiate the production of antibodies (42). 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, 42). 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 (9). Similarly, the secretion of IL-4 from T_{H2} cells promotes the differentiation of additional T_{H2} cells (22, 54). In the case of the T_{H2} subset, IL-4 can synergize with IL-2 with respect to growth, which greatly enhances T cell expansion and effectiveness (58).

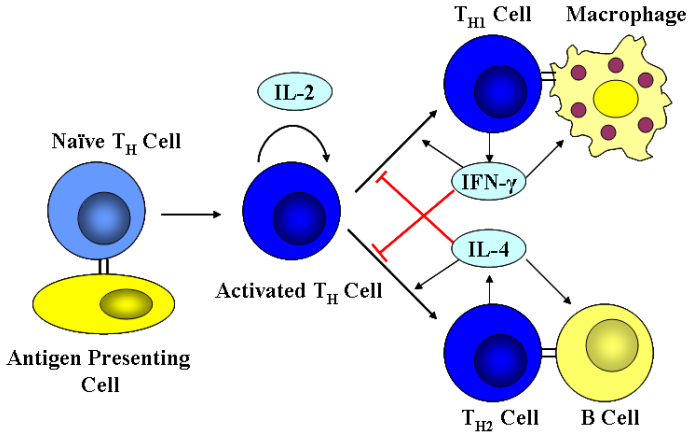

Figure 1.2 Cytokine induced T_H cell differentiation After a mature, yet naïve T_H cell encounters its specific antigen it secretes IL-2, inducing rapid proliferation. Once primed, a T_H cell can differentiate into either a T_{H1} or a T_{H2} subset, responsible for stimulating the development of either 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.

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 (25, 51).

At the same time, the macrophages activated by T_{H1} cells and the massive amounts of antibodies secreted by the B lymphocytes 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 (6).

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 its 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 (44). Because IL-2 is critical for the clonal expansion of T cells, it is also commonly referred to as T cell growth factor (29). 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 (32). 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 (13).

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 (29). In contrast, the IL-2Rβ (p75) and γ_c (p64) chains together are both necessary and sufficient to initiate the signaling cascades (38).

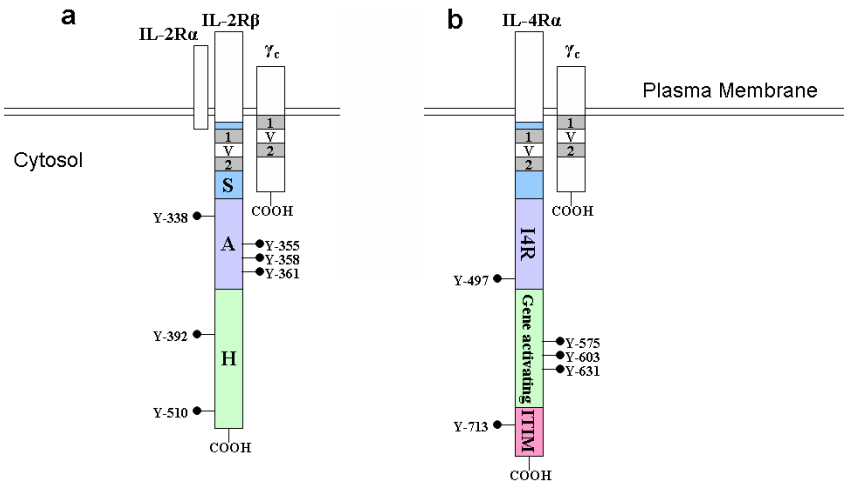

Figure 1.3 The IL-2 and IL-4 receptor complexes a) The IL-2R is composed of three subunits: the IL-2Rα, the IL-2Rβ and γ_c. Only the IL-2Rβ and γ_c chains participate in signal transduction, with each containing a Box 1 and Box 2 motif to which Jak binds. The IL-2Rβ can be functionally broken down into 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. Five conserved tyrosine residues necessary for signal transduction exist within the IL-4Rα.

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 (32). 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 a low level of signaling (29). 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 (15, 29, 32).

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

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 (44). 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 (41, 52).

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 (19). 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 domain (A region), comprised of amino acids 313-382. This is followed by the carboxyl terminal proline-rich region (H region), which includes amino acids 378-525 (18, 19). IL-2Rβ mutants lacking each of these domains 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 domain was required for cell proliferation, and the A section was necessary for activation of tyrosine kinases such as Lck and Fyn (11, 30, 33). 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 (43). 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 (38, 40). Many growth factor receptors have intrinsic tyrosine kinases, which upon ligand addition auto-activate and phosphorylate the receptor (55). This however, 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 (16, 28). Being in close proximity, the Jak kinases are able to sequentially transactivate each other via tyrosine phosphorylation (68). 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 (13, 14) *(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 activate further signaling proteins that may bind to IL-2Rβ (30, 34, 35, 61). 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 (13); 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 (7).

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 (36, 37). 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 (3). 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, perfectly illustrates why it is critical to maintain a proper balance of cytokines within the body (5, 64).

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 (36). This attraction is responsible for the extremely high affinity observed for IL-4Rα and its ligand (K_d = 20 pM) (39).

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 (45).

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 (39) *(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 (60). 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 (49). 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 (60). 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 (21, 39). The IL-4R is unique among cytokines and growth factors in the respect that it does not incite the Ras/Erk signaling cascade, as will be further discussed in Chapter 2 (47, 53, 66, 67).

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 these cytokines are simultaneously exposed to T cells,

they exert a synergistic effect that enhances the rapid expansion of the target cells (48, 63). Synergy may 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 the presence of an antagonistic relationship at high ligand levels. It is believed that this modification is in part due to the downregulation in functional IL-2R complexes by IL-4, owing to the fact that both cytokines utilize the γ_c subunit (4, 10).

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CHAPTER 2

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

2.1 BACKGROUND

The IL-2 receptor mediates three important signaling pathways: 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 in that it does not employ the Ras/Erk cascade. In this chapter each of these cascades is discussed with regard to the proteins involved, activation and regulation mechanisms and downstream targets. Figure 2.1 summarizes the pathways induced by IL-2 and IL-4 in T cells and illustrates how sequential activation of signaling proteins leads to clonal expansion.

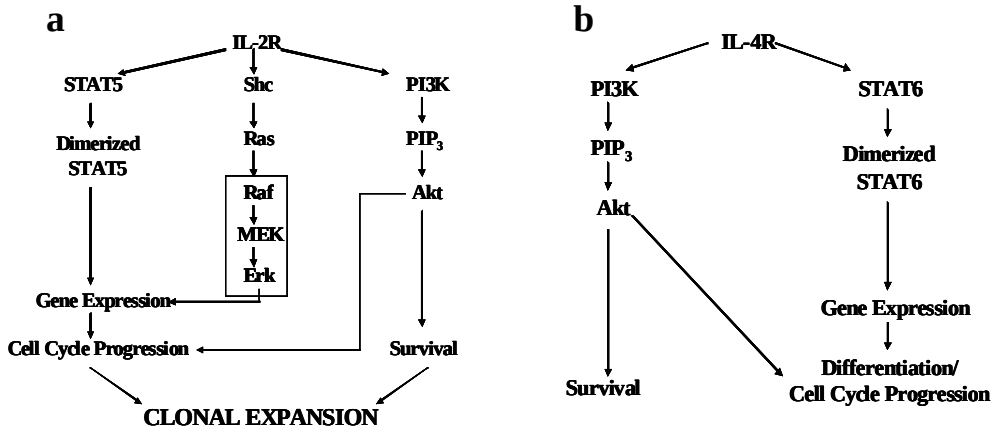

Figure 2.1 IL-2 and IL-4 induced signaling pathways a) Following IL-2/IL-2R ligation, three signaling cascades are activated – the Jak/STAT5, the Ras/Erk and the PI3K/Akt pathways. These pathways cooperate to induce the cell cycle progression and survival necessary to achieve rapid proliferation and differentiation, also known as clonal expansion. **b)** Following activation of the IL-4R, two major signaling pathways are activated leading to cell cycle progression, differentiation and survival – the Jak/STAT6 and the PI3K/Akt cascades. IL-4 is unique among cytokines in the respect that it does not employ the Ras/Erk cascade.

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 phosphotyrosine sites on cytokine receptors, become activated via tyrosine phosphorylation by Jaks, dimerize and translocate to the nucleus, where they induce gene expression (31, 66, 67). STATs are also capable of triggering the activation of additional transcription factors, promoting progression through the cell cycle and initiating differentiation (112). The Jak/STAT pathway induces several negative regulatory mechanisms, 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 the number of 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 these 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 representing 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 a phosphotyrosine residue, 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 may bind to the receptor.

Another responsibility of the Jak family is the phosphorylation of the receptor-bound STAT transcription factors. STATs are the preferential substrate of Jaks, and will be phosphorylated by these specific kinases before any other proteins present (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 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 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 only required for maximal transcriptional activity. Serine phosphorylation of the STAT dimers is thought 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-stimulated cell cycle progression and proliferation (153).

Following activation of the IL-2 receptor complex, STAT5 is recruited from the cytosol and binds to IL-2Rβ through one of two specific phosphotyrosine sites, Y-392 or 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 DNA motifs (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. Gene activation 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. Translation of several proteins that are required for the proliferative response can also be induced by STAT5, 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 the 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 necessary binding sites for NFAT, NFκB, STAT6 and *c-maf*. Additionally, the IL-4 3' enhancer region specifically requires GATA-3 and NFAT association 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 T lymphocytes. It has been demonstrated that dimerized 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). The control

of the signaling and gene induction following Jak/STAT6 activation is crucial in the regulation of T cell growth and differentiation in the presence of IL-4.

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 following 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 attract 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 phosphotyrosine 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) *(Figure 2.2)*. Although PI3K preferentially catalyzes the phosphorylation of PI(4,5)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 between PH domains and 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.

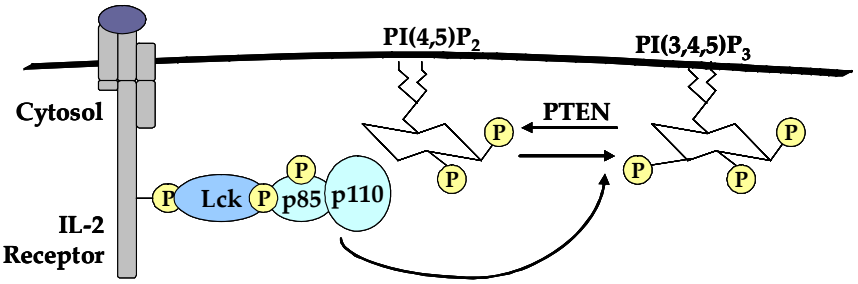

Figure 2.2 PI3K activation by the IL-2 receptor system The p85 regulatory subunit of class IA PI3Ks associates with the IL-2 receptor complex through the utilization of a non-receptor kinase, such as Lck. The p110 catalytic subunit of PI3K catalyzes the reaction of $PI(4,5)P_2$ to $PI(3,4,5)P_3$ in the plasma membrane The creation of the lipid messenger PIP_3 is highly regulated by the phosphatase PTEN.

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 the levels of both active 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) *(Figure 2.2).* 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 requires a serine phosphorylation 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 further 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 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 isolated 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 induction of 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, Akt dependent activation of NFκB, which 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 stimulate proliferation by promoting cell cycle progression. 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 T-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 targets 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 simultaneously (40, 77, 125). Once Shc is bound the IL-2Rβ, 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 constitutively 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 GEF into close proximity with the membrane-bound Ras-GDP, promoting the formation of Ras-GTP (39, 50, 88, 119) *(Figure 2.3)*.

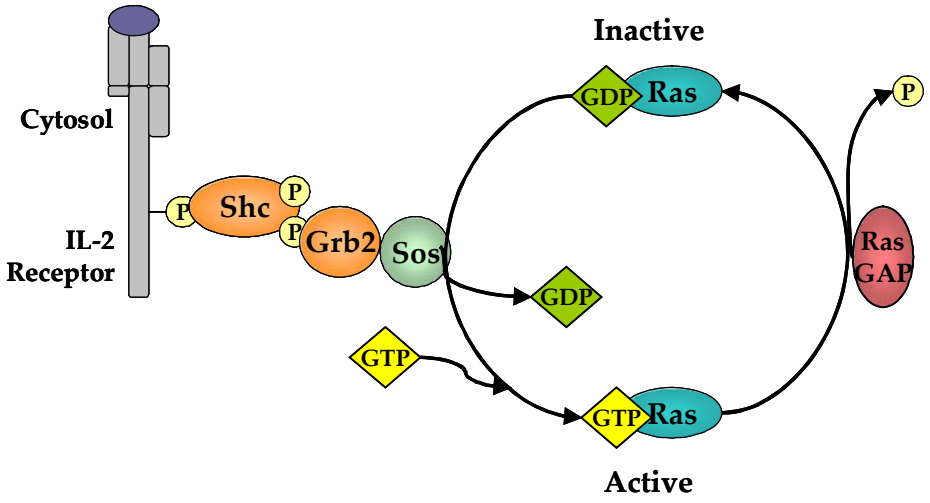

Figure 2.3 IL-2 induced activation and regulation of Ras Following IL-2 receptor activation the receptor-bound adapter protein Shc couples with the Grb2/Sos dimer. Sos, a known GEF, dislodges the bound GDP from the inactive Ras, which allows for rapid loading of GTP, stimulating Ras activation. RasGAP proteins regulate the level of functional Ras-GTP by stimulating the hydrolysis of GTP to GDP, thus returning Ras to its inactive GDP-bound state.

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). After SHP-2 binds either directly to Shc or indirectly through the adapter protein Gab2, it becomes activated by a two-step mechanism. 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 and initiating its 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 various 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 require phosphorylation by Raf 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 of the serine/threonine kinase Erk. Erk is highly regulated and requires both tyrosine and threonine phosphorylation to be operative, specifically at sites Y-185 and T-183 (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*, (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 the hydrolysis of GTP to GDP and the inactivation of Ras (146, 148, 157) *(Figure 2.3).* 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. Together, 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 discussed (43, 110).

Additionally, the signaling proteins in one pathway have the potential to laterally modulate proteins in other pathways, altering the dynamics and balance of the signal transduction network *(Figure 2.4)*. One striking instance of crosstalk in the IL-2 signal network 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, and thus activating PI3K (130, 141). The adapter protein Shc can also activate both Ras and PI3K simultaneously, with the aid of adapter proteins Grb2 and Gab2 (58, 60, 115). The co-localization of these pathways is the perfect opportunity for Ras to bind p110 and positively modulate the PI3K pathway.

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 the serine phosphorylation of STAT dimers 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 can stabilize their association and help 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 adapter protein 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 conflicting positive and negative effects on Ras/Erk pathway activation. With multiple pathways that can potentially both positively and negatively modulate each other, an alteration in the balance of the signaling network, due to crosstalk, may be a realistic explanation behind the IL-2/IL-4-induced synergistic growth response.

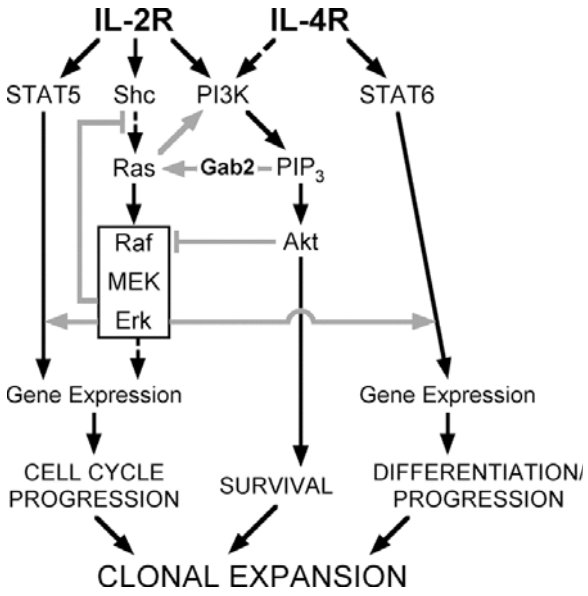

Figure 2.4 Crosstalk in the IL-2/IL-4 system There are several possible crosstalk pathways occurring the IL-2/IL-4 co-stimulation system. Erk has the potential to serine phosphorylate the STAT dimers, increasing their stability. Ras is also able to positively modulate the activation of PI3K by phosphorylating the catalytic domain. Following the creating of PIP_3 at the membrane by PI3K, Gab2 will bind to it and recruit Grb2/Sos, which in turn can activate Ras away from the receptor complex. It has been demonstrated that Akt will 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

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CHAPTER 3

CHARACTERIZATION OF SIGNALING PATHWAYS THAT GOVERN T CELL PROLIFERATION IN RESPONSE TO INTERLEUKIN-2 AND INTERLEUKIN-4

3.1 INTRODUCTION

Cells sense and respond to chemical and physical stimuli through signal transduction networks, which mediate cell proliferation, differentiation, migration, and survival (32). Interleukin-2 (IL-2) and interleukin-4 (IL-4) are key regulators of the adaptive immune response, controlling T cell expansion and differentiation (31, 36). It has been reported that IL-2 and IL-4 co-stimulation induces T cell proliferation in a synergistic manner; at lower concentrations of IL-2 and IL-4, the collective proliferation rate is greater than the additive effects elicited by each cytokine alone (61, 71). At the level of intracellular signaling networks, the basis for this synergy is unknown.

Signaling events stimulated by IL-2 are mediated by its high affinity receptor complex, composed of the IL-2Rα, IL-2Rβ and γ_c subunits (39, 54). IL-4 binds to a dimeric receptor, consisting of the IL-4Rα and γ_c (56). Assembly of these high-affinity complexes is a multi-step process, whereby the common γ_c subunit is recruited after the initial binding of the cytokine, a requirement for initiation of signaling (55, 65). By the same token, γ_c recruitment stabilizes cytokine/receptor association. Thus, to the extent that IL-2 and IL-4 receptors compete for a limited pool of γ_c, the two cytokines are expected to antagonize one another. Such antagonism has been observed, but it is apparently uni-directional; IL-4 causes partial inhibition of IL-2 binding, whereas IL-4 binding is unaffected by the presence of IL-2 (11, 18).

As described in Chapter 2, IL-2 and IL-4 receptors activate both common and unique signaling pathways. IL-2 receptors activate the Ras/extracellular signal-regulated kinase (Erk), phosphoinositide 3-kinase (PI3K)/Akt and Janus kinase (Jak)/STAT5 pathways (6,

20). IL-4 is unique among cytokines in that it does not induce Ras/Erk; it does, however, activate the PI3K/Akt pathway as well as a distinct Jak/STAT6 cascade (30, 56, 75).

Following IL-2 or IL-4 addition, the kinases Jak1 and Jak3 are recruited and bind to the receptor complexes, where they phosphorylate specific, conserved tyrosine residues on the IL-2Rβ or the IL-4Rα (34, 37). STAT5 and STAT6 are the primary downstream proteins targeted for phosphorylation by Jak1 following their association with the IL-2β and the IL-4Rα, respectively (35, 51, 76). Phosphorylation of these transcription factors results in reciprocal dimerization of the STATS, translocation of STAT dimers to the nucleus, and DNA binding (14, 33).

The lipid and serine/threonine kinase PI3K, comprised of a p85 regulatory domain and a p110 catalytic domain, is activated following treatment with either IL-2 or IL-4 and is responsible for cell proliferation and survival (19, 77). After cytokine stimulation, PI3K is recruited to and associates with the receptor after which the necessary conformational changes occur for activation (26, 53). Active PI3K produces 3' phosphoinositide (PI) lipids [$PI(3,4,5)P_3$ and $PI(3,4)P_2$] that act as second messengers, recruiting downstream signaling proteins to the plasma membrane (48, 72). One 3' PI-dependent target is the serine/threonine kinase Akt, which mediates numerous pro-survival and cell cycle progression pathways (1, 7, 10).

The Ras/Erk cascade is a well characterized signaling pathway stimulated by most growth factors and cytokines to control cell proliferation and differentiation (25, 49). Phosphorylation of the IL-2R complex provides a binding site for, and promotes the assembly of, the Shc/Grb2/Sos complex, which controls activation of Ras at the plasma membrane (50, 58). Ras then triggers a signaling cascade of protein kinases that include Raf, MEK, and Erk, which are regulated by phosphorylation (4, 40, 59). Once active, Erk translocates to the nucleus where it phosphorylates transcription factors such as *c-fos*, *c-jun*, Elk-1 and *c-myc* (12, 23, 60).

Although many studies have contributed to the knowledge of the roles of signaling proteins in T cell growth, little has been done in the analysis of these proteins following co-stimulation with IL-2 and IL-4. We have sought to characterize signal transduction pathways activated by IL-2 and IL-4 receptors in the HT-2 cell line, a helper 2 (T_{H2}) T cell line derived from mouse. This cell line is an ideal model because it constitutively expresses IL-2 and IL-4 receptors, does not normally make its own IL-2 or IL-4, and proliferates in response to IL-2 (29, 43). We show that IL-2-stimulated HT-2 cell proliferation in static culture is affected by IL-4 in two distinct ways. IL-4 antagonizes proliferation at early times, effectively prolonging the lag phase, yet on the other hand it delays the onset of stationary phase and allows a higher cell density to be achieved.

In conjunction with these growth studies, activation levels of key signaling proteins were measured following IL-2 and IL-4 addition – specifically Akt, Erk, STAT5 and STAT6. At low but not high concentrations of IL-2, IL-4 partially inhibits IL-2 induced activation of Akt, Erk and STAT5, consistent with the aforementioned antagonism of IL-2 binding and saturation of the intracellular signaling machinery. This effect cannot completely explain the inhibition of IL-2-stimulated proliferation, however, because the IL-4-induced extension of lag phase is observed at higher concentrations of IL-2. IL-2-stimulated activation of Akt, Erk, and STAT5 was found to be transient, subject to depletion of IL-2 from the medium, and IL-4 does not extend the duration of those signals. By comparison, IL-4-stimulated activation of STAT6 is sustained, and we speculate that this unique pathway is responsible for most if not all of the effects of IL-4 in these cells.

3.2 MATERIALS AND METHODS

Cell culture and reagents

Human recombinant IL-2 and murine recombinant IL-4 were acquired from Peprotech. Other cell culture reagents were purchased from Invitrogen. The murine T helper cell line HT-2 was obtained from American Type Culture Collection. 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 (47, 74). The cells were subcultured in 75 cm^2 T flasks in an incubator at 37 ºC and 5% CO_2.

Antibodies against phospho-specific Akt (pSer 473), phospho-specific p44/p42 MAPK (pThr 202/pTyr 204), phospho-specific STAT5 (pTyr 694), total STAT5, phospho-specific STAT6 (pTyr 641) and total STAT6 were from Cell Signaling Technology. Antibodies against total Akt 1/2 and total Erk-1 were from Santa Cruz Biotechnology. Anti-rabbit and anti-goat secondary antibodies were acquired from Cell Signaling Technology and Sigma, respectively. The pharmacological inhibitors U0126, PD98059, LY303511 and LY294002 were purchased from Calbiochem. Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific.

Growth curve analysis

HT-2 proliferation analyses were performed in non-treated, 47 mm plates. Briefly, cells were seeded at $3x10^4$ cells/mL, in a total volume of 3 mL, with medium containing the indicated cytokine concentrations. Approximately every 12 hours, sampling was performed by scraping, mixing and counting the number of cells within each plate using a Z_1 Coulter-Particle Counter (Beckman Coulter).

Preparation of detergent lysates

For short-term stimulation experiments, HT-2 cells were incubated in cytokine-free media for 4 hours prior to cytokine addition 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 $2.5x10^5$ cells/mL. For each lysate, 12 mL of cell culture was removed and treated with appropriate cytokines; at the pre-determined time, the cells were 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) (28). All chemicals were obtained from either Fisher Scientific or Sigma. Cells were vortexed and incubated on ice for 20 minutes, then centrifuged at 6000xg at 4 ºC for 15 minutes to remove cellular debris. The supernatants were collected and transferred to a fresh tube, and the lysates were stored at –20 ºC.

For long-term stimulation, 30 mL of cells were incubated in a 15 cm non-treated petri dish at an initial density of $3x10^4$ cells/mL with media containing the indicated cytokine concentrations. Prior to lysis, each plates was scraped with a cell lifter to remove any adherent cells from the surface; the cells were mixed and counted to determine cell density. Next, 25 mL of cells were pooled and lysed by the method described above.

Adhesion studies

In non-treated, 10 cm petri dishes, 10 mL of cells were incubated at a density of $3x10^4$ cells/mL for IL-2/IL-4 combined treatment and at a density of $1x10^5$ cells/mL for IL-4 only stimulation. The higher initial cell density for the IL-4-only scenario compensates for the lack of cellular proliferation in the absence of IL-2. At each time point, the numbers of cells in both the supernatant and attached to the plate were assessed using the Z_1 Coulter-Particle Counter.

Experiments were also performed to determine if, upon removal of IL-4, the adhered cells would detach. The above experimental setup was repeated, and the cells were given four days to fully adhere. The medium was removed and replaced with medium containing 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 as described above.

Quantitative Western blotting

Pooled cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). 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 (44, 69). 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 determined by normalizing the amount of phosphorylated protein by the total amount of that protein, quantified in parallel.

3.3 RESULTS

The main objective of this work was to characterize the responses of HT-2 cells to IL-2, IL-4 and IL-2/IL-4 co-stimulation, both in terms of cell responses such as proliferation and at the level of intracellular signal transduction pathways. Four major signaling pathways have been implicated in the IL-2-/IL-4-receptor network, and one end-point protein was selected to represent the activity of each: Akt, Erk, STAT5 and STAT6. We hoped to correlate inducible changes in signaling behavior to any observed alterations in HT-2 growth patterns following IL-2/IL-4 co-stimulation.

3.3.1 Characterization of HT-2 cell proliferation and intracellular signaling stimulated by IL-2: effect of IL-2 dose.

The kinetics of HT-2 cell proliferation, measured as a function of time in static culture, were evaluated for various initial concentrations of IL-2 *(Figure 3.1 A).* Not surprisingly, these kinetics exhibit a lag phase in the first 12 hours or so. Thereafter, the population density increases at a rate that depends on the initial concentration of IL-2, with saturation of the apparent rate at IL-2 concentrations of ~ 50 pM and above. The lower range of IL-2 concentrations, which represent plausible biological levels (21, 39), produce a definite growth response without saturating the high-affinity receptors. At later times, a plateau in cell density is observed, and its onset is a function of IL-2 dose; concentrations significantly greater than 50 pM continue to yield gains in the duration of robust cell growth. These kinetics are characteristic of the well-known mechanism by which receptor-mediated endocytosis mediates clearance of IL-2 and other ligands from the medium over time (68).

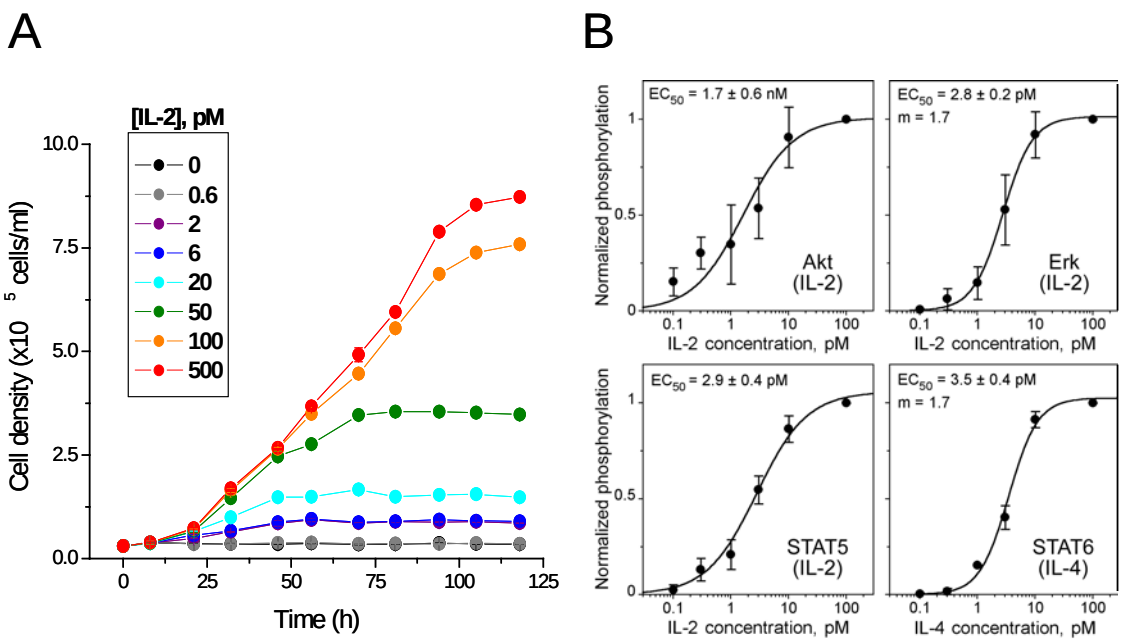

Figure 3.1 Growth and signaling dose response of T cells following cytokine stimulation **A)** HT-2 cells were incubated with varying IL-2 concentrations to characterize the proliferative response of the cell line. The cell density was determined in triplicate (represented here as mean ± S.E.), and the data are representative of two independent experiments. **B)** The activation profiles of key proteins were monitored by quantitative immunoblotting as a function of cytokine dose; the stimulation time was 1 hour. In three independent experiments, IL-2-stimulated activation of Akt, Erk and STAT5 was measured in triplicate (mean ± S.E., n=3). IL-4-stimulated STAT6 activation was measured in triplicate in two independent experiments (mean ± S.E., n=2). In each experiment, the basal rate was subtracted, and the stimulated levels were normalized by the maximum value. The normalized dose response, y, was fit to the Hill function: $y = y_{max} * C^m / \{EC_{50}{}^m + C^m\}$, where C is the concentration of cytokine and y_{max}, EC_{50}, and m are fit parameters. The exponent m was constrained so that only values greater than or equal to 1 were allowed; m = 1 except where noted.

Next, dose response curves were constructed for the activation of Akt, Erk and STAT5 stimulated by IL-2 and of STAT6 stimulated by IL-4 *(Figure 3.1 B)*. Estimated values of the EC_{50}, the concentration of cytokine that elicits half-maximal activation, were in the range of ~ 2–3 pM for all responses. Considering the reported K_D values of IL-2 and IL-4 in the range of 10–50 pM, the results suggest that receptor-mediated signaling through these pathways is subject to saturation, perhaps at the level of a common signaling component. This would be consistent with recruitment of a limiting pool of Jak1 or Jak3, for example.

Based on the results thus far, it is clear that signal transduction readouts in HT-2 cells, measured at quasi-steady state after a relatively short period of stimulation, do not correlate quantitatively with IL-2-stimulated proliferation. An IL-2 concentrations of ~ 15–20 pM is sufficient to saturate the initial signaling responses but not the rate of cell proliferation prior to the onset of stationary phase. This observation is entirely consistent with the now classic conceptual model of IL-2 receptor signaling, which underscored the importance of the duration of signaling, and not only its peak magnitude, as a key determinant of T cell proliferation (45).

3.3.2 IL-4 initially antagonizes IL-2-stimulated signaling and proliferation of HT-2 cells but also fosters higher maximal cell densities in static culture.

At the level of stimulated DNA synthesis or cell proliferation, both synergistic and antagonistic effects of IL-2 and IL-4 co-stimulation have been reported (11, 61). Analysis of the kinetics of HT-2 cell proliferation revealed aspects of both such effects under static culture conditions, in an IL-4 dose-dependent manner (*Figure 3.2 A&B*). IL-4 initially antagonizes IL-2-stimulated HT-2 cell proliferation, delaying the emergence of the culture from lag phase. On the other hand, IL-4 also delays the onset of stationary phase, prolonging cell growth such that a higher cell density is achieved compared with IL-2 alone. These qualitative features are observed at a concentration of IL-2 close to the EC_{50} for signal transduction (2 pM; *Fig. 3.2 A*) as well as at a significantly higher IL-2 dose (15 pM; Fig. 3.2 *B*). In the absence of cytokines, HT-2 cells do not proliferate and eventually undergo apoptosis. Additionally, it was confirmed that IL-4 alone does not elicit significant HT-2 cell proliferation.

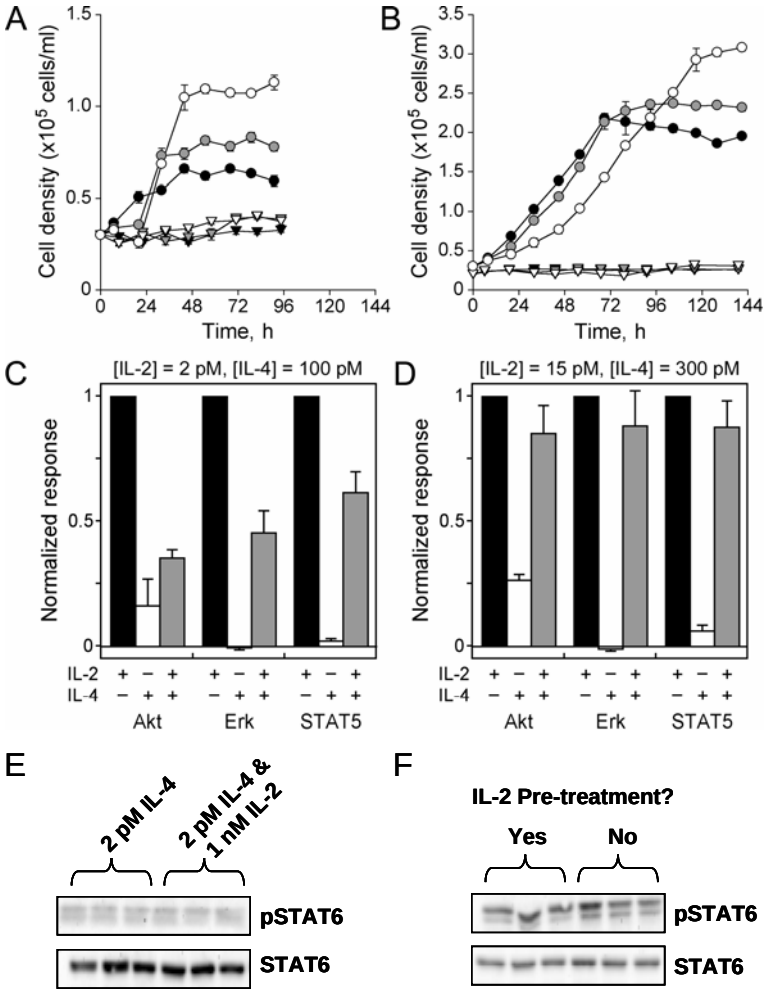

Figure 3.2 The response of HT-2 cells to IL-2/IL-4 co-stimulation A-B) Growth kinetics of HT-2 cells were monitored following stimulation with IL-2 only, IL-4 only or combinations of IL-2 and IL-4. The cell density was measured in triplicate (mean ± S.E.) and the displayed data are representative of three independent experiments. **A)** −▼− no cytokines, −▼− 2 pM IL-4, −▽− 100 pM IL-4, −●− 2 pM IL-2, −●− 2 pM IL-2/2 pM IL-4, −○− 2 pM IL-2/100 pM IL-4 **B)** −▼− no cytokines, −▼− 10 pM IL-4, −▽− 300 pM IL-4, −●− 15 pM IL-2, −●− 15 pM IL-2/10 pM IL-4, −○− 15 pM IL-2/300 pM IL-4 **C&D)** In order to determine early signaling pathway activation in the presence of IL-2 and IL-4, experimental matrices were designed: one with a sub-saturating dose of 2 pM IL-2 (mean ± S.E., $n = 3$) and the second with a saturating concentration of 15 pM IL-2 (mean ± S.E., $n = 4$). Cells were treated with the indicated doses of IL-2 and/or IL-4 for 1 hour, lysed and analyzed in triplicate. **E)** HT-2 cells were treated with 2 pM IL-4, alone or in combination with 1 nM IL-2, for one hour. Cell lysates were probed for STAT6 activation. **F)** For the pre-treatment samples, HT-2 cells were stimulated with 1 nM IL-2 for 24 hours prior to the 1 hour stimulation with 2 pM IL-4. The other samples underwent concurrent stimulation of 1 nM IL-2 and 2 pM IL-4 for 1 hour. Active STAT6 levels are compared between the two circumstances.

In an effort to determine how IL-4 delays the onset of IL-2-stimulated HT-2 cell growth, various combinations of the two cytokines were administered, and Akt, Erk, and STAT5 phosphorylation measurements were conducted after 1 hour of stimulation. These measurements were designed to capture the initial signaling burst that is thought to be required for cell cycle progression (38). At a sub-saturating dose of 2 pM IL-2, a high dose of IL-4 (100 pM) partially inhibits all of the IL-2-stimulated pathways measured (*Figure 3.2 C*). The degree of inhibition, ranging from approximately ~ 30–70%, is consistent which the reported antagonism of IL-2/IL-2R binding (5, 11). Of the signaling readouts tested, the observation that Akt activation was subject to the greatest degree of inhibition is at least qualitatively consistent with the notion that IL-2 and IL-4 receptors compete not only for γ_c, Jak1, and Jak3, but presumably also for recruitment of PI3K and other components required for activation of Akt. At a higher dose of 15 pM IL-2, at which IL-4 still effectively antagonizes IL-2 receptor binding (11), the antagonism of IL-2-stimulated Akt, Erk, and STAT5 signaling is significantly ameliorated (*Figure 3.2 D*). This is not inconsistent with the postulated competition mechanism outlined above, because the signaling pathways are saturated at this IL-2 dose (*Fig. 3.1 B*); thus, a partial reduction in the number of activated IL-2 receptors is expected to have only a modest effect.

Consistent with the inability of IL-2 to antagonize IL-4/IL-4R binding (52), it was confirmed that IL-4-stimulated STAT6 phosphorylation, assessed at a half-maximal concentration of 2 pM IL-4, is unaffected by the presence of a saturating IL-2 dose *(Figure 3.2 E)*. An alternative possibility, based on the observation that chronic IL-2 stimulation of T cells results in significant downregulation of the γ_c subunit (67), is that IL-2 stimulation might indirectly affect IL-4 receptor signaling over time. This possibility was ruled out, as pretreatment with 1 nM IL-2 for 24 hours had no effect on IL-4-stimulated STAT6 phosphorylation (*Figure 3.2 F*). Apparently, any γ_c subunit downregulation was not of a sufficient magnitude to impact IL-4 receptor signaling in our cells, and it is concluded that IL-4 stimulation of STAT6 activation is effectively independent of IL-2 receptor signaling.

Comparing these data, the initial inhibition of IL-2-stimulated HT-2 cell proliferation by IL-4 cannot be entirely explained by the inhibition of Akt, Erk, and STAT5 signaling, because this effect on proliferation is apparent even at an IL-2 concentration (15 pM) that fosters maximal signaling in the presence of IL-4 (*Fig. 3.2 B & D*). One possibility is that at least one signaling pathway required for IL-2-stimulated proliferation is not saturated at this dose and is therefore more strongly affected by antagonism of IL-2/IL-2R. An alternative and perhaps more likely possibility is that a pathway unique to the IL-4 receptor, such as STAT6, antagonizes or otherwise stalls the sequence of molecular processes required for the onset of cell growth.

3.3.3 IL-2/IL-4 co-stimulation of T cells yields transient activation of Akt, Erk and STAT5 coupled with sustained STAT6 signaling.

Given the established importance of the duration of IL-2 receptor-mediated signaling in T cell proliferation (66), we sought to characterize the kinetics of IL-2-stimulated signaling and the influence of IL-4 on those kinetics *(Figure 3.3)*. At the same sub-saturating dose of 2 pM IL-2 used above, phosphorylation of Akt, Erk, STAT5 and STAT6 reached peak levels within ~ 30 minutes and decayed thereafter. Within ~ 6 hours, IL-2-stimulated Erk and STAT5 phosphorylation had decayed to approximated basal levels, whereas Akt phosphorylation was somewhat more sustained (*Fig. 3.3*), consistent with its lower EC_{50} concentration (*Fig. 3.1 B*). The presence of 100 pM IL-4, mirroring the conditions used above (*Fig. 3.2 A & C*), resulted in the partial inhibition of peak signaling as expected; furthermore, the presence of IL-4 did not prolong the time course of IL-2-stimulated signaling (*Fig. 3.3*). By comparison with IL-2 stimulated signaling, IL-4-stimulated STAT6 phosphorylation was sustained throughout the time course.

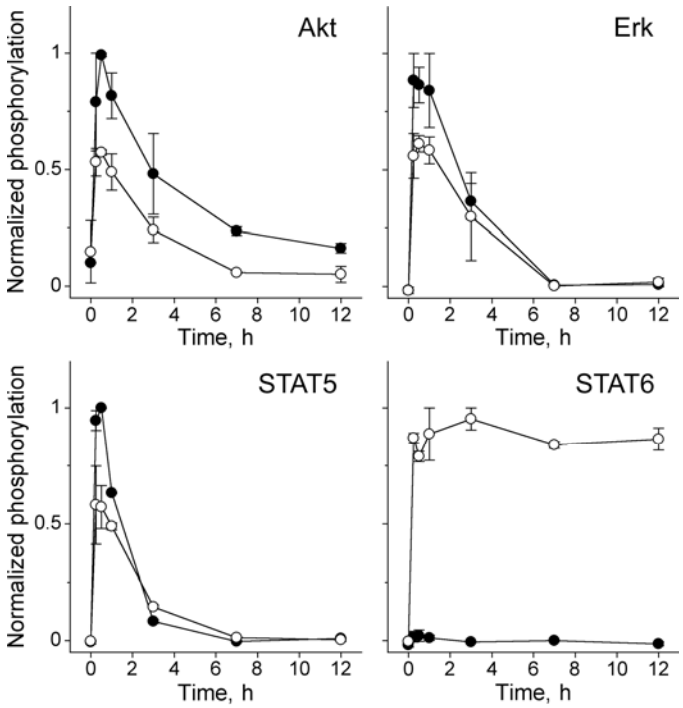

Figure 3.3 Signaling duration following treatment with 2 pM IL-2 HT-2 cells were dosed with either 2 pM IL-2 (−●−) or 2 pM IL-2 and 100 pM IL-4 (−○−). In two independent experiments, samples were collected over a 12 hour time course and analyzed in triplicate to determine the signaling kinetics of Akt, Erk, STAT5 and STAT6 (mean ± S.E., n = 2).

Distinct mechanisms that might explain the transient nature of the IL-2-induced signals were considered. The first is that low concentrations of IL-2 (e.g., 2 pM) might be depleted from the extracellular medium, as a consequence of receptor-mediated endocytosis or the action of cell-derived proteases. This mechanism was deemed highly plausible based on our interpretation of the proliferation dose response presented in *Fig. 3.1 A*. Another common consequence of receptor-mediated endocytosis is downregulation of receptors from the cell surface, but this mechanism is not expected when the ligand concentration is well below the effective K_D of high-affinity receptor binding (46). Another plausible mechanism is an adaptive response at the level of signal transduction, typically achieved through negative feedback in the system. The ligand depletion mechanism was tested by re-feeding the cells with 2 pM IL-2 after the signaling levels have decayed to near basal levels (t = 6 hours); upon re-feeding, the IL-2-stimulated levels of phosphorylated Erk and STAT5 rebounded to

near peak levels (*Figure 3.4 A*), confirming that ligand depletion is at least a major contributor to, if not the root cause of, the decay in IL-2 receptor-mediated signaling.

As a follow-up experiment, HT-2 proliferation was monitored after re-stimulation with 2 pM IL-2; if ligand depletion is significant as asserted above, one would expect IL-2 re-feeding to induce a second round of proliferation beyond the stationary phase otherwise observed. Indeed, this was found to be the case *(Figure 3.4 B)*. Together, these results indicate that transient IL-2 signaling in static culture is caused at least in part by a ligand depletion effect.

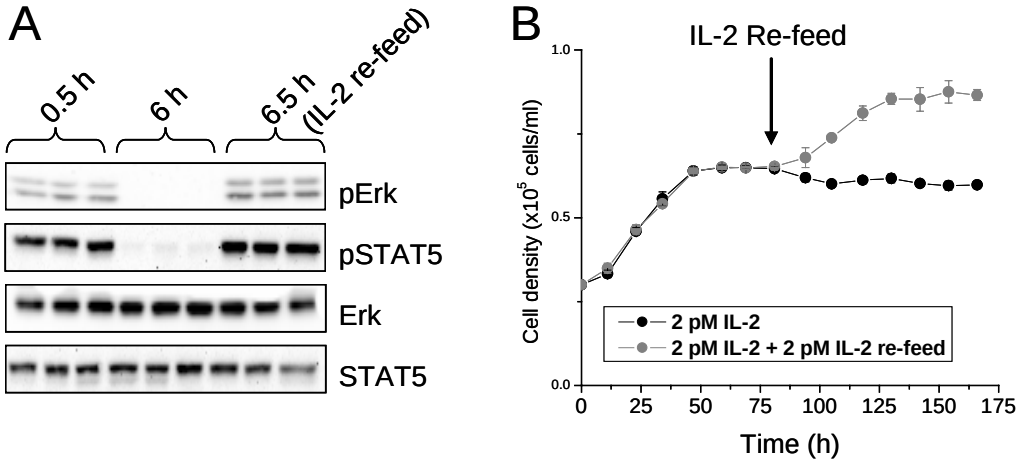

Figure 3.4 Transient IL-2 signaling and HT-2 growth recover following cytokine re-feed A) HT-2 cells were dosed with 2 pM IL-2, and samples were collected for analysis at 30 minutes and 6 hours post-stimulation. At 6 hours, the remaining cells were resuspended in fresh medium containing 2 pM IL-2 and lysed after 30 minutes. All time points were collected in triplicate, and phosphorylation of Erk and STAT5 was quantified by immunoblotting. The blots shown are representative of two independent experiments. **B)** Two HT-2 cultures were each treated with 2 pM IL-2, and their cell densities were monitored as a function of time. After reaching stationary phase, one culture was resuspended in fresh medium containing 2 pM IL-2, whereas the second culture was left undisturbed. The proliferative response was measured in triplicate (reported here as mean ± S.E.), and the data shown here are representative of two independent experiments.

Kinetic analysis of signaling readouts induced by a higher dose of IL-2 (15 pM), in the absence or presence of a high IL-4 concentration, yielded results that were consistent with those outlined above (*Figure 3.5*). As expected, IL-2-stimulated phosphorylation levels of Akt, Erk, and STAT5 are more prolonged than for the lower IL-2 dose, yet the signaling

readouts still decay to near basal levels within ~ 48 hours. Here, as in the case of 2 pM IL-2 stimulation, the presence of IL-4 does not enhance the persistence of the IL-2-stimulated signals. By comparison, in the presence of IL-4, STAT6 phosphorylation is sustained over the course of several days (*Fig. 3.5*). At a lower, sub-saturating concentration of IL-4 (10 pM), the magnitude of the response is lower yet similarly sustained, indicating that IL-4-stimulated signaling, at least through STAT6, is not subject to the same ligand depletion effect affecting IL-2 receptor-mediated pathways.

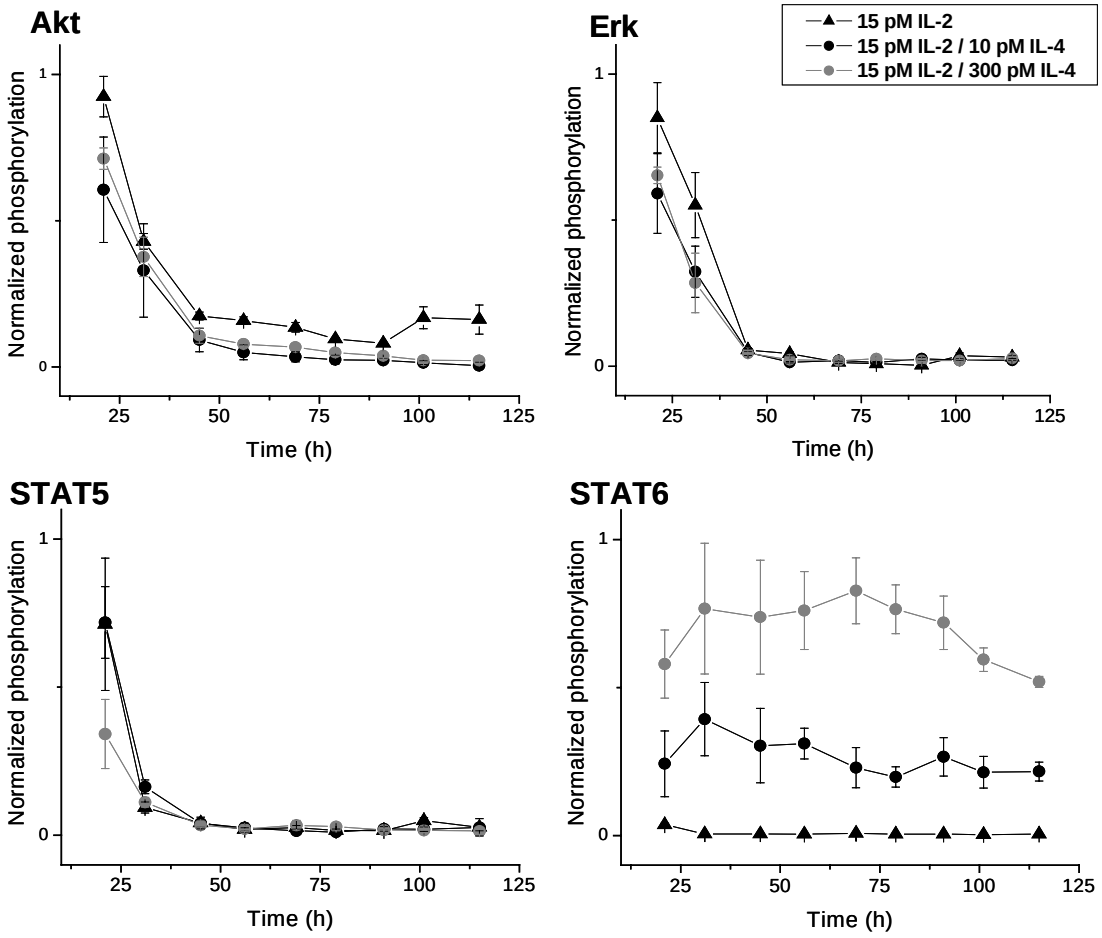

Figure 3.5 Transient signaling in response to 15 pM IL-2 HT-2 cells were stimulated with either 15 pM IL-2 alone or in combination with 10 pM or 300 pM IL-4. Phosphorylation of Akt, Erk, STAT5 and STAT6 were measured by quantitative immunoblotting (mean ± S.E.).

3.3.4 IL-2-stimulated HT-2 cell proliferation is perturbed by pharmacological inhibitors of intracellular signaling pathways.

To further elucidate the roles of the PI3K/Akt and the Ras/Erk signaling pathways in IL-2-induced T cell proliferation, we assessed the sensitivity of HT-2 cell growth kinetics to pharmacological inhibition of PI3K and MEK, the kinases most proximal to Akt and Erk, respectively. The inhibitors used are LY294002 in the case of PI3K (57, 73) and both PD098059 (3, 27) and U0126 (17, 62) in the case of MEK. It was verified that proper concentrations of these pharmacological inhibitors block their intended targets in HT-2 cells *(Figure 3.6 A)*; PI3K-dependent Akt phosphorylation induced by IL-2 is effectively blocked in the presence of 50 μM LY294002, and both 50 μM PD098059 and 10 μM U0126 are effective in preventing IL-2-stimulated Erk phosphorylation.

Testing the effects of these inhibitors on HT-2 cell proliferation revealed differential sensitivity to the inhibitors of PI3K- and MEK-dependent signaling (*Figure 3.6 B*). Whereas the PI3K inhibitor LY294002 completely abrogates IL-2-stimulated proliferation, inhibition of MEK/Erk has a significant but less severe effect by comparison.

The dramatic effect of LY294002 on HT-2 cell growth is potentially problematic in light of recent studies showing that this compound is less selective than previously assumed (9, 13, 15, 22). A control compound, LY303511, which differs from LY294002 by an amine substitution of its morpholine oxygen group, does not inhibit PI3Ks (16), yet it inhibits several of the known unintended targets of LY294002, including mTOR, CK2, GSK3β, and MCP-1 (13, 22, 42). Given these pharmacological profiles, comparison of IL-2-stimulated HT-2 cell proliferation in the presence of LY294002 or LY303511 suggests that inhibition of targets other than PI3K severely impacts HT-2 cell growth, but proliferation is blocked completely when PI3K is inhibited *(Figure 3.6 C)*.

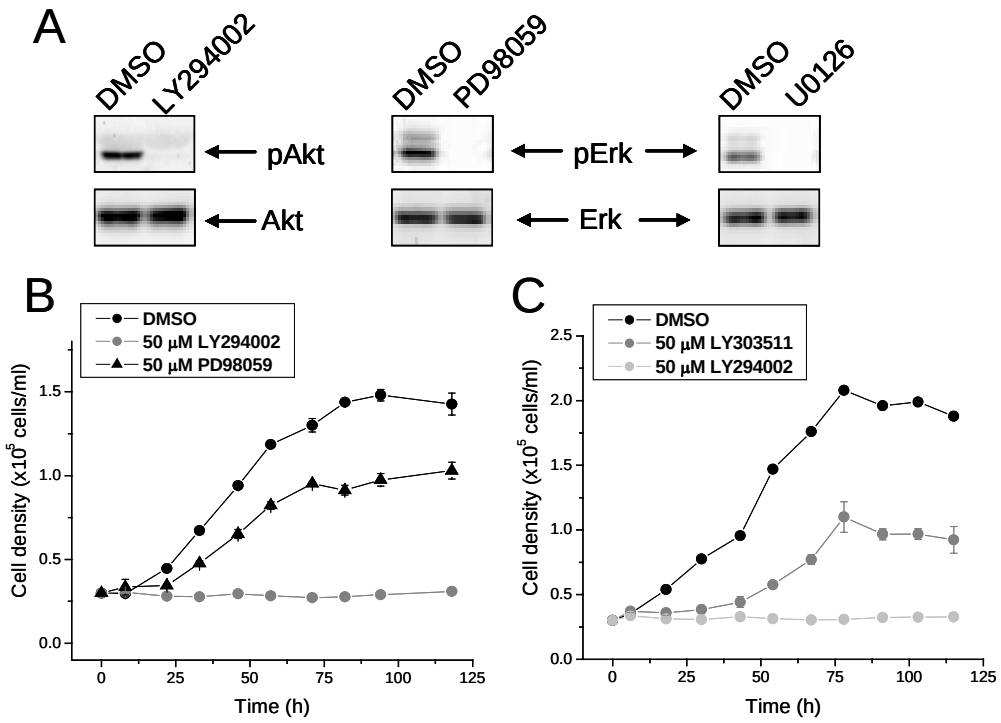

Figure 3.6 Pharmacological inhibitors and their effect on HT-2 signaling and growth **A)** HT-2 cells were pre-treated with the indicated pharmacological inhibitor, as described in the main text, or DMSO as a vehicle only control. Following stimulation with IL-2, cells were lysed and target signaling activation was determined in triplicate. Representative blots are shown demonstrating the intended effects of the inhibitors. **B)** HT-2 cells were pre-treated with DMSO, LY294002 or PD098059 prior to addition of 15 pM IL-2 and the resulting growth was observed. The cell density was measured in triplicate (represented here as mean ± S.E.), and the data are representative of two independent experiments. **C)** HT-2 cells were pre-treated with either LY294002 or LY303511 then stimulated with 15 pM IL-2. The proliferative response was determined and compared for these inhibitors against a DMSO control. Cell growth was monitored in triplicate (reported here as mean ± S.E.), and the data shown are representative of two independent experiments.

Other experiments were performed to assess the effect of the timing of signaling pathway inhibition (*Figure 3.7*). Administering inhibitors after 24 hours of 15 pM IL-2 stimulation *(Fig. 3.7 A)* did not prevent inhibition of IL-2-stimulated proliferation in these experiments, but the interpretation of these results is somewhat uncertain, as the kinetics presented in Fig. 3.5 indicate that signaling levels are still elevated after 24 hours under these conditions.

Experiments were also performed to assess whether or not MEK/Erk inhibition would significantly impact IL-2-stimulated growth after the observed transient of Erk phosphorylation was completed (*Fig. 3.7 B*). As expected, the results show that MEK inhibition has an increasingly diminished effect on HT-2 cell proliferation as the staggering of IL-2 and inhibitor addition becomes longer.

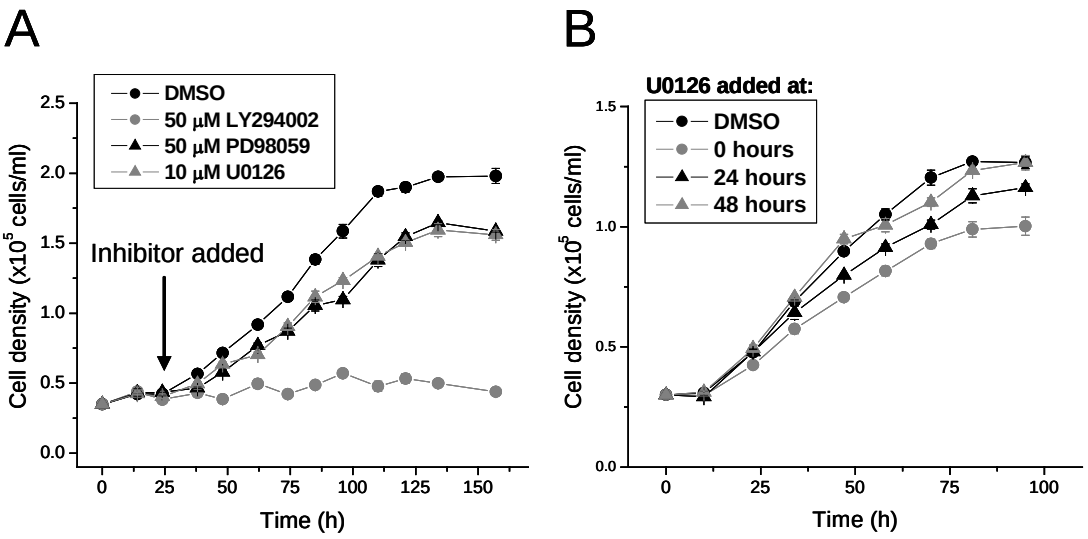

Figure 3.7 Growth analysis following inhibitor treatment at various times A) Cells were stimulated with 15 pM IL-2 at the beginning of the growth studies. After 24 hours, cells were treated with either 50 μM LY294002, 50 μM PD098059 or 10 μM U0126 and the resulting growth was monitored in triplicate (mean ± S.E.). **B)** 10 μM of U0126 was added to HT-2 cells stimulated with 15 pM IL-2 at either 0, 24 or 48 hours. The ensuing growth patterns were recorded in triplicate (reported here as mean ± S.E.) and compared to cells treated with DMSO only.

3.3.5 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 several surface receptor molecules, which mediate T cell adhesion and migration (8, 41, 70). We observed that following treatment with IL-4 HT-2 cells, which normally are in suspension, began adhering to non-treated petri dishes *(Figure 3.8 A-B)*. In addition to this effect, the cellular phenotype was altered when exposed to IL-4: cells became significantly larger while some started producing cellular extensions *(Figure 3.8 C-D)*.

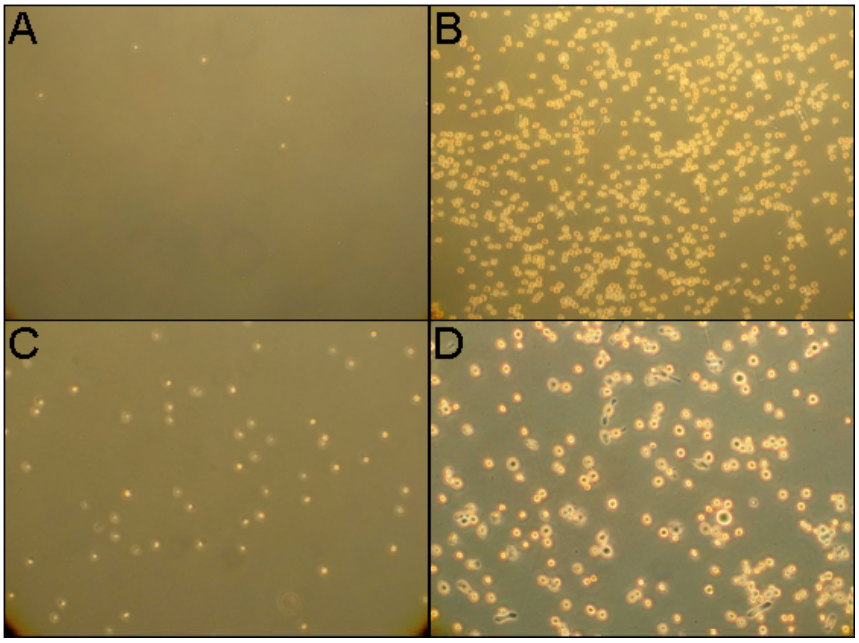

Figure 3.8 IL-4 provokes HT-2 cells to adhere and undergo phenotypic alterations A) Following treatment of HT-2 cells with IL-2, the plate was washed and imaged (10x), displaying no cellular adhesion. **B)** Even after washing, cells undergoing concurrent IL-2 and IL-4 stimulation remain strongly attached to the plate surface (10x). **C)** When cultured with IL-2, HT-2 cells are small, round and in suspension (20x). **D)** After IL-4 exposure, the cell phenotype is altered, becoming larger, producing cellular extensions and adhering (20x).

Next, the extent of HT-2 adhesion was assessed following co-stimulation with IL-2 and IL-4. It was found that the number of HT-2 cells attaching to a non-treated, polystyrene plate increased in an IL-4 dose-dependent fashion with time *(Figure 3.9 A)*. 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. To verify that this observed phenomenon was induced by IL-4, experiments were performed in which cellular adhesion was quantified in the presence of IL-4 only *(Figure 3.9 B)*. The observed trends following exposure to 10 pM and 100 pM IL-4 were remarkably similar to the parallel experiments containing IL-2, suggesting that HT-2 adhesion is solely dependent upon IL-4.

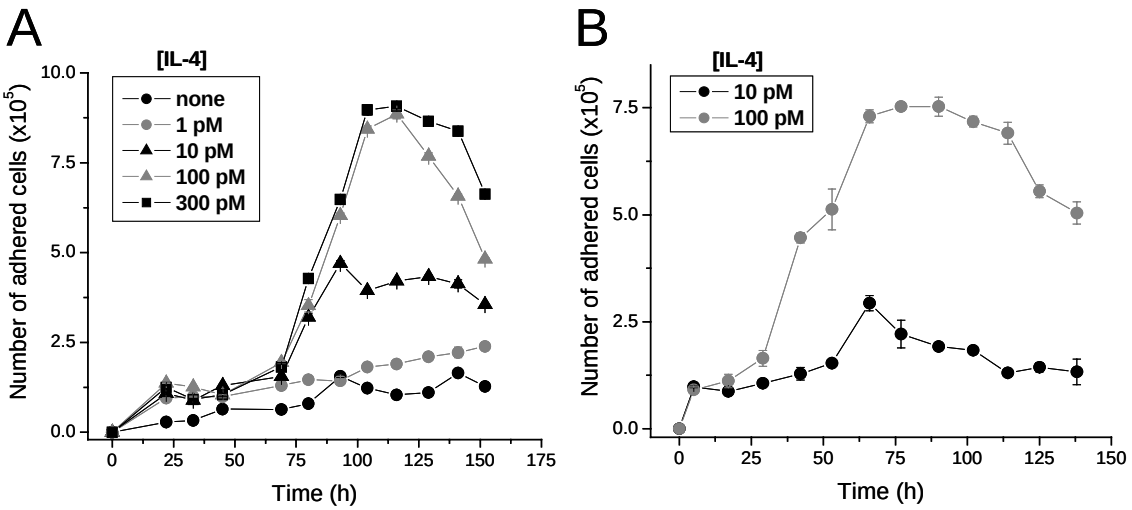

Figure 3.9 Quantification of IL-4-induced adhesion A) Following IL-2 and IL-4 addition the number of cells that adhered to a 10 cm non-treated petri dish was monitored over time in triplicate (mean ± S.E.). **B)** Cells were treated with IL-4 only and resulting cellular adhesion was analyzed in triplicate (mean ± S.E.).

To further investigate this adhesive response, we tested to see if continuous IL-4 stimulation was required to maintain HT-2 cell adhesion *(Figure 3.10)*. Cells were pretreated under the same IL-2/IL-4 co-stimulatory conditions used in the above adhesion studies, allowing adequate time for cell attachment to occur. The IL-4-supplemented media was then removed and replaced with fresh media containing only IL-2. The number of attached cells was monitored over several days; after approximately 4 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.

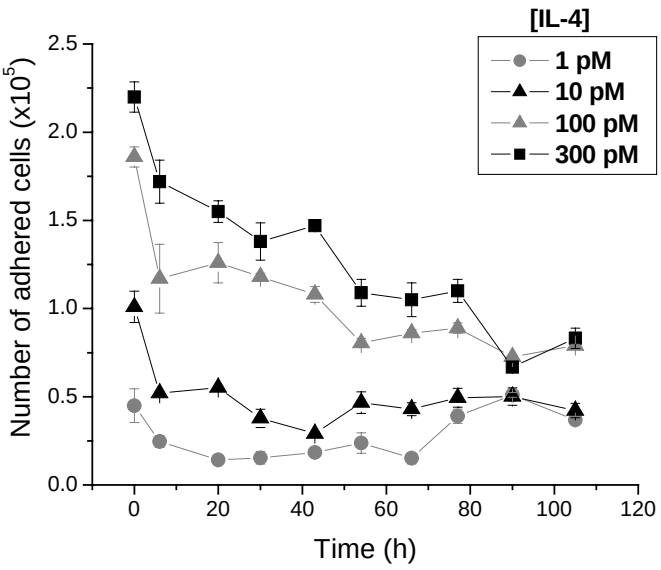

Figure 3.10 Plated HT-2 cells begin to detach following IL-4 removal Cells were allowed to adhere for 4 days in the presence of the cytokines indicated, after which they were incubated with 15 pM IL-2 only and the extent of cellular adhesion was quantified over time (adhered cells measured in triplicate and shown as mean ± S.E.).

3.4 DISCUSSION

In this study, we assessed the functional responses and signal transduction stimulated by IL-2 in T cells, 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 initially delays and then later enhances the rate of IL-2 stimulated HT-2 proliferation. Accordingly, IL-4 inhibits IL-2 stimulation of certain transient signaling intermediates while inducing sustained activation of IL-4-dependent proteins. Although great headway was made, additional work will need to be performed to fully elucidate the mechanisms involved in the cellular response to concurrent IL-2 and IL-4 stimulation.

Potential role and mechanism of adhesion

Our work demonstrated that HT-2 cells undergo a strong adhesive response when stimulated with IL-4. It is well documented that IL-4 induces expression of several surface molecules on T cells, resulting in cellular adhesion (8, 41, 70). It will be beneficial to uncover which surface molecule 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 is present in the medium or is being secreted by the HT-2 cells. A likely candidate would be an exopolysaccharide that is recognized by IL-4-induced cell surface molecules (64).

Along the same line of thought, it would be advantageous to establish the role that cell adhesion plays in IL-4 induced proliferation. We surmise that IL-4-stimulated adhesion is a critical step that T cells must undergo before synergy can occur. It would be interesting to see if the synergistic growth response to IL-2/IL-4 co-stimulation would still occur if HT-2 adhesion was blocked, possibly through the utilization of non-adhesive plate coatings. The next logical question that follows is whether STAT6 is responsible for this behavior in HT-2 cells. After blocking its effects, possibly through a siRNA approach (63), it could be easily determined if STAT6 is required for IL-4-dependent cell adhesion.

Crucial signaling contributions to IL-2-dependent T cell growth

The results obtained from the pharmacological inhibitor experiments help to further elucidate the role of signaling in IL-2-stimulated growth of T cells. These studies revealed a dependency on both the PI3K/Akt and the Ras/Erk pathways for optimal HT-2 cell proliferation in the presence of IL-2. Separating the PI3K contributions from LY294002 experimentation can be complicated because recent studies have shown that the compound is less selective than previously assumed. LY303511 is a control substance that does not inhibit PI3Ks, but does inhibit several of the known, unintended targets of LY294002 (13, 22, 42). Comparison of IL-2-stimulated HT-2 cell growth in the presence of LY294002 or LY303511 *(Figure 3.6)* demonstrates that inhibition of targets other than PI3K severely impacts HT-2 cell growth, while inhibiting PI3K completely blocks proliferation. Inhibitor studies also verified the importance of early IL-2 signaling events in the HT-2 proliferative response. As expected, we demonstrated that MEK inhibition, from U0126 treatment, has an increasingly diminished effect on HT-2 cell growth as the length of time between IL-2 and inhibitor addition was elongated.

Sub-saturating signaling from the IL-2 receptor allows for a greater synergistic response in the presence of IL-4

It has been reported that T cells will undergo a stronger synergistic growth response in the presence of IL-4 when stimulated with sub-saturating concentrations of IL-2 (11). To ascertain if this held true for our cellular system, the 2 pM IL-2 growth experiments were performed concurrently with the 15 pM doses to allow for direct evaluation. By comparing the differences in final cell densities, it was shown that lowering the IL-2 concentration to a half-maximal value increased the IL-4-driven synergistic effect. For 2 pM IL-2, addition of IL-4 caused an approximate increase in cell growth of 56%, whereas this increase was reduced to 31% for 15 pM IL-2 (*Table 3.1)*. Further confirmation of this phenomenon was discovered when no IL-4-dependent synergy was observed following HT-2 growth in the presence of 1 nM IL-2 (data not shown).

Table 3.1 Synergy comparison between 2 pM and 15 pM IL-2 (mean ± S.E.)

		2 pM IL-2			15 pM IL-2	
		IL-2 only	*IL-2 + high dose IL-4*		*IL-2 only*	*IL-2 + high dose IL-4*
Trial 1	**Cell Density**	6.50E+04	1.10E+05		1.40E+05	1.75E+05
	% Increase	69%			25%	
Trial 2	**Cell Density**	1.00E+05	1.42E+05		2.20E+05	3.00E+05
	% Increase	42%			36%	
Average % Increase		56% ± 13%			31% ± 5%	

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. This assumption also appears to extend to the signaling initiated by IL-2 and IL-4, which demonstrate EC_{50} values lower than expected *(Figure 3.1B)* suggesting that receptor is subject to saturation. Indeed, this effect could be initiated by a limitation of γ_c subunits, but is also consistent with a competition over available Jak1 and/or Jak3: all of which are required to initiate signaling from the IL-2 and IL-4 receptors.

Following co-stimulation of a sub-saturating dose of IL-2 (2 pM) with IL-4, it was shown that IL-4 partially inhibits all the IL-2-stimulated pathways measured. This inhibition, ranging from 30%-70%, is consistent with the reported antagonism of the IL-2/IL-2R in the presence of IL-4. The short-term analysis of Akt activation showed the most pronounced decrease in activation following co-stimulation, even though both IL-2 and IL-4 activate the PI3K/Akt pathway. These results indicates that in addition to competition for γ_c and Jak1/Jak3, it is probable that there is a limitation of PI3K or another compound required for Akt activation. The results obtained from IL-2/IL-4 co-stimulation with 15 pM IL-2 *(Figure 3.2D)*, which saturates the IL-2 signaling response, are also consistent with this hypothesized competition effect. In this situation, it was expected that the partial reduction of activated IL-2 receptors, induced by the introduction of IL-4, would have only a modest effect on the IL-2-dependent signaling.

The impact of IL-2 on IL-4-dependent STAT6 activation was also scrutinized. This work presented evidence that IL-4 is able to out-compete IL-2 for the limiting γ_c subunit. Theoretically, cells pretreated with IL-2 should occupy most of the available γ_c receptors prior to the addition of IL-4. Since IL-2-pre-treatment studies indicated no negative effect on STAT6 activation *(Figure 3.2F)*, it would imply that IL-4 is able to displace the pre-bound ligand and introduce competition between the IL-2 and IL-4 receptors. Additionally, research performed in this study has verified that IL-4 stimulation of STAT6 is effectively independent of IL-2 receptor signaling.

The initial antagonism of HT-2 growth by IL-4 cannot be fully explained by the corresponding inhibition of Akt, Erk and STAT5. In the presence of a saturating dose of IL-2 (15 pM) significant growth inhibition is observed accompanied by only a modest downregulation of IL-2-dependent signaling. It is possible that another signaling pathway required for IL-2-dependent growth is not saturated following stimulation with 15 pM IL-2 and is severely antagonized in the presence of IL-4. Alternatively, a pathway unique to the IL-4 receptor, most probably STAT6, may be able to negatively regulate IL-2-driven HT-2 proliferation.

IL-2 signaling transience is brought on by ligand depletion

We sought to characterize the kinetics of target signaling proteins following stimulation with 2 pM IL-2. Studies showed that IL-2-dependent signaling was transient by nature, whereas IL-4 signaling was sustained. The activation levels of Akt, Erk and STAT5 peaked at 30 minutes post cytokine addition followed by a steady decline in signaling intensity. Erk and STAT5 signals were returned to basal levels within 6 hours, whereas the Akt response was somewhat more sustained. The IL-2 signaling kinetics in the presence of IL-4 mirrored IL-2 and were accompanied by the expected inhibition of Akt, Erk and STAT5. Interestingly, IL-4 neither prolonged nor shortened the duration of IL-2-dependent signaling.

One mechanism that might explain the IL-2 signaling transience is that that low concentrations of IL-2 might be depleted from the extracellular medium over time. Receptor

bound ligand could be internalized though receptor-mediated endocytosis followed by degradation in intracellular lysosomes (2). This mechanism of significant ligand depletion was deemed most plausible based on the IL-2 dose dependent growth profiles of HT-2 cells *(Figure 3.1A)*. The IL-2 re-feed experiments *(Figure 3.4)* verified that the IL-2 transient signaling is at least partially explained by significant ligand depletion. This supposition was further proven by the kinetics analysis following HT-2 treatment with 15 pM IL-2, which demonstrated transient signaling of Akt, Erk and STAT5 lasting approximately 48 hours. The prolonged duration of IL-2 signaling is directly proportional to the increase in initial IL-2 concentration, when compared against the 2 pM IL-2 kinetics. Assuming a constant rate of receptor-mediated endocytosis and ligand degradation, these results are consistent with IL-2 depletion as the root of signaling transience.

Synergistic growth and the possible role of STAT6

Studies have revealed the existence of a synergistic effect on T cell growth following co-stimulation with IL-2 and IL-4. The activation of Akt, Erk and STAT5 were shown to be transient and therefore have no direct link to the synergy growth phase. In contrast, STAT6 activation remained high throughout the time course in an IL-4 dose-dependent manner *(Figure 3.5)*. These results suggest that IL-4 signaling, at least through STAT6, is not plagued by the same ligand depletion that occurs in the IL-2 receptor system. 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 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. Other signaling proteins that could be targeted include transcription factors downstream of STAT6, such as GATA-3, NFAT and NFκB (24). One study that would shed more light on the role of STAT6 in T cell growth is to knock down its expression through a siRNA approach (63). After removing its effects 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.

The completion of this work has great potential in augmenting the understanding of how T cell clonal expansion occurs following co-stimulation with IL-2 and IL-4. This knowledge could be of vast importance in the research to ward off cancer and infectious diseases. If continued, this work could easily be expanded to encompass other cell lineages and cytokines to help further understand and bolster the immune response.

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