THE ROLE OF PARALLEL SIGNALING PATHWAYS IN MAINTAINING LYMPHOID CELL SURVIVAL

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II. ABSTRACT

T lymphocytes are critical for a functional immune system. However, their uncontrolled activation and growth can be manifested as a number of lymphoid derived diseases such as autoimmunity, allograft rejection, and lymphoma. Therefore, understanding the role of key signaling molecules and the ability to modify their activity in *mature cells* may provide new therapeutic strategies to treat T cell mediated disorders. The purpose of this study was to dissect the role of key signaling pathways activated by interleukin-2 (IL2) and other T cell growth factors that share the IL2 receptor gamma chain (γc) (IL2, 4, 7, 9, 13, 15, 21) in mediating T cell growth and survival. To accomplish this objective, antisense oligonucleotides targeted against signaling molecules including γc, Raf isoforms, Stat3, traditional pharmaceuticals to disrupt Janus tyrosine kinase 3, PI3K (wortmannin), mTor (rapamycin) and MEK kinase (PD98059) activity were utilized and their effects characterized in lymphoid cells. Antisense oligonucleotide targeted against γc inhibited protein expression (40%) in a lymphoid tumor cell line inducing apoptotic death and cell cycle arrest in G2-M phase, as measured by FACS analysis of Annexin V and Propidium Iodide stained cells. Inhibition of Jak3 (PNU156804) showed similar effects and disrupted Stat5 and MAPK activation. Novel evidence is shown here that IL2 can activate multiple isoforms of Raf, and inhibition of these isoforms by antisense oligonucleotides failed to affect cell survival or Stat5 activation. Similar results were found for inhibition of PI3K, mTor and MEK kinases. Lastly, we show that Stat3 antisense oligonucleotide treatment of a human tumor cell line bearing constitutively activated Stat3 induces cell death (40%, measured with TUNEL assay) but that IL2 can rescue these cells possibly mediated via Stat5 that becomes hyperactivated in Stat3 deleted cells. Evidence is provided that suggests Bcl2 plays key role in this cell survival process. We conclude from our findings that the Mapk, Stat and PI3K pathways likely function as separate entities in lymphoid cells, suggesting that evolutionarily these pathways might have evolved to perform specialized cell functions that may be redundant for other T cell activities. Moreover, our results suggest that Stat molecules are critical to the survival and growth of T cells and represent a therapeutically relevant target to combat T cell derived disorders.

III. ABBREVIATIONS

AS ODN	antisense oligodeoxynucleotide
DBD	DNA Binding Domain
EDTA	Ethylene-diamine-tetraacetic acid
EGF	Epidermal Growth Factor
EMSA	Electromobility Shift Assay
Erk	Extracellular Regulated Kinase
FACS	Fluorescence Activated Cell Sorter
FCS	Fetal Calf Serum
FERM	Four point one, ezrin, radixin and moesin homology domain
GAPDH	Glycerinaldehide 3-phosphate dehidrogenase
GAS	Gamma Activated Sequence
GST	Glutathione S-transferase
IL	Interleukin
IL2Rγ	IL2 Receptor Gamma Chain (γc)
Jak	Janus tyrosine kinase
LIF	Leukemia Inhibitory Factor
Mapk	Mitogen Activated Protein Kinase
MCM5	Minichromosome Maintenance 5
Mek	Map kinase/Erk kinase
Mm Ctrl	Mismatched Control
mTor	mammalian target of rapamycin
ODN	Oligodeoxynucleotide
OSM	Oncostatin M
PAGE	Polyacrylamide Gel Electrophoresis
PDGF	Platelet Derived Growth Factor
PHA	Phytohemagglutinin
PI3K	Phosphatidylinositol 3-kinase
PIAS	Protein Inhibitor of Activated Stat
PKC	Protein Kinase C

PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PMSP	proline-methionine-serine-proline
PSP	proline-serine-proline
PVDF	polyvinylidene fluoride
PXSP	proline-X-serine-proline
PY	phosphotyrosine
RAPA	rapamycin
Scr Ctrl	Scrambled Control
SDS	Sodium Dodecyl Sulphate
SH	Src homology
SHP	SH2 containing protein tyrosine phosphatase
SOCS	Suppressor of Cytokine Signaling
SOS	Son of Sevenless
Stat	Signal Transducer and Activator of Transcription
TAD	Transactivation Domain
TCGF	T cell growth factor
Th1/2	T helper 1/2
Tris	2-amino-2-hydroxymethylpropane-1, 3-diol
TUNEL	TdT-mediated dUTP-X Nick End Labeling
Wort	wortmannin

IV. INTRODUCTION

The objective of this study is to investigate the contribution of IL2-induced alternate signaling pathways to control lymphoid cell growth and survival. IL2 is a major regulator of immune homeostasis in T lymphocytes, mediating cell proliferation, survival, apoptosis and differentiation. However, uncontrolled T cell function and activation is the underlying abnormality in a number of immune mediated disorders like allergy, leukemia, lymphoma, allograft rejection and autoimmunity. Understanding the role of key pathways critical to T cell growth and survival will contribute to the therapy of immune mediated diseases. IL2 activates a number of pathways including Jak1/3, Stat5a/b and 3, Syk and Lck, Mitogen Activated Protein Kinase (Mapk), PI3K and mTor. This work attempts to dissect the role of these pathways in mediating lymphoid cell growth and survival. The ***rationale*** behind the proposed research centers on compelling new data demonstrating that mice deficient in γc, Jak3 and Stat5 transcription factors are immune suppressed [1-5]. To accomplish the objective of this proposal, we will test whether targeted inhibition of cell signaling components related to the Jak/Stat signaling pathway blocks T cell activity.
The **hypothesis** to be tested is that Stat molecules are critical in maintaining T cell viability via the following specific aims:

(1) Determine whether selective disruption of γc protein expression or Jak3 activity inhibits lymphoid/T cell function.
(2) Determine whether disruption of PI3K, Mapk and Raf pathways blocks Stat5 serine kinase and lymphoid cell growth.
(3) Determine whether constitutively active Stat3 provides a survival pathway in a lymphoid tumor cell line.

At the conclusion of this research we expect to have established that the relative importance of several molecules activated within the Jak3 signaling pathway are critical for lymphoid cell activity. In addition to providing novel insight into normal physiologic and host defensive processes in which these pathways play a critical role in T cell activity, we expect that this work will facilitate a greater understanding of the pathogenesis of T cell

mediated diseases. This work is important because new therapeutic strategies could result and directly benefit to the treatment of these disorders.

1. T cell activation in response to IL2

T lymphocytes (T cells) are a major component of adaptive immunity whereby antigens are recognized and eradicated in an antigen specific manner [6]. A T cell, which has recognized an antigen, will secrete interleukin-2 (IL2). IL2 - a key T cell growth factor - drives clonal expansion of antigen activated T lymphocytes. T lymphocytes therefore play a central role in normal immune activation against pathogens, but when become unregulated they display immunity against self antigens to promote autoimmunity, allergy, and graft rejection. Therefore, a major challenge in immunology is to better understand essential signal transduction pathways mediating T cell activation. By identifying these regulatory pathways, specific pharmaceutical and biological modifiers may be designed as therapeutic modalities to combat these immune mediated diseases.

As stated above, IL2 is a cytokine produced by activated T lymphocytes, and also is important for immune homeostasis [6]. Mice deficient in IL2 or IL2 receptors share a common phenotype of autoimmunity and accumulation of activated T lymphocytes [6]. Therefore, IL2 plays both a positive and a negative role in immune activation. The IL2R complex comprised of three distinct single membrane spanning receptors IL2Rα, IL2Rβ, and IL2Rγ. IL2 binds to the heterotrimeric receptor complex with a high affinity (K_d 10-80 pM). Binding of IL2 to its receptor complex results in activation of tyrosine kinases Jak1, Jak3, Syk and Lck [6]. Activated Jak1 and Jak3 phosphorylate the IL2Rβ chain at specific tyrosine residues. The phosphorylated tyrosine residues serve as docking sites for other signaling molecules with phosphotyrosine binding domains such as Shc, PI3K, Stat5, Stat3 and others, which link the IL2/IL2R to the downstream signaling events (reviewed in [7]). The following is a summary of our current knowledge on the IL2 activated pathways and their physiological role in T cells (Figure 1).

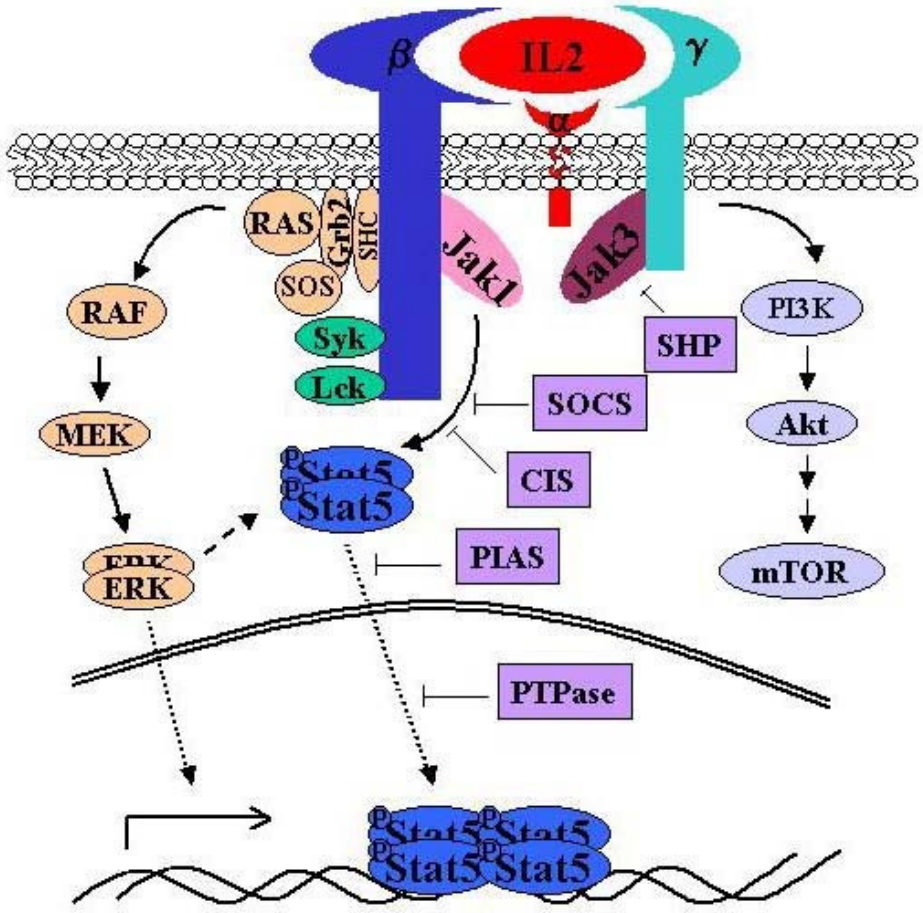


Figure 1. Schematic model of IL2 induced pathways. Binding of IL2 to its receptor chains, IL2Rα, β, and γ promotes the recruitment and activation of signaling proteins such as Jak1, Jak3, Syk, Shc, Lck, PI3K, and Stat5. PIAS (Protein Inhibitors of Activated Stats), and SOCS (Suppressors of Cytokine Signaling) are negative regulators of Stat and Jak proteins, respectively.

1.1. Activation of Jak3/Stat5a/b

1.1.1. Biology of Jaks There are four mammalian Jaks: Jak1, Jak2, Jak3, and Tyk2. Jak3 expression is limited to NK cells, thymocytes, B cells and myeloid cells [8]. The other three members, Jak1, Jak2 and Tyk2 are more widely expressed. Janus kinases are unique among the other tyrosine kinases in having tandem kinase and pseudokinase domains. Figure 2A is a schematic diagram of Jak kinases.

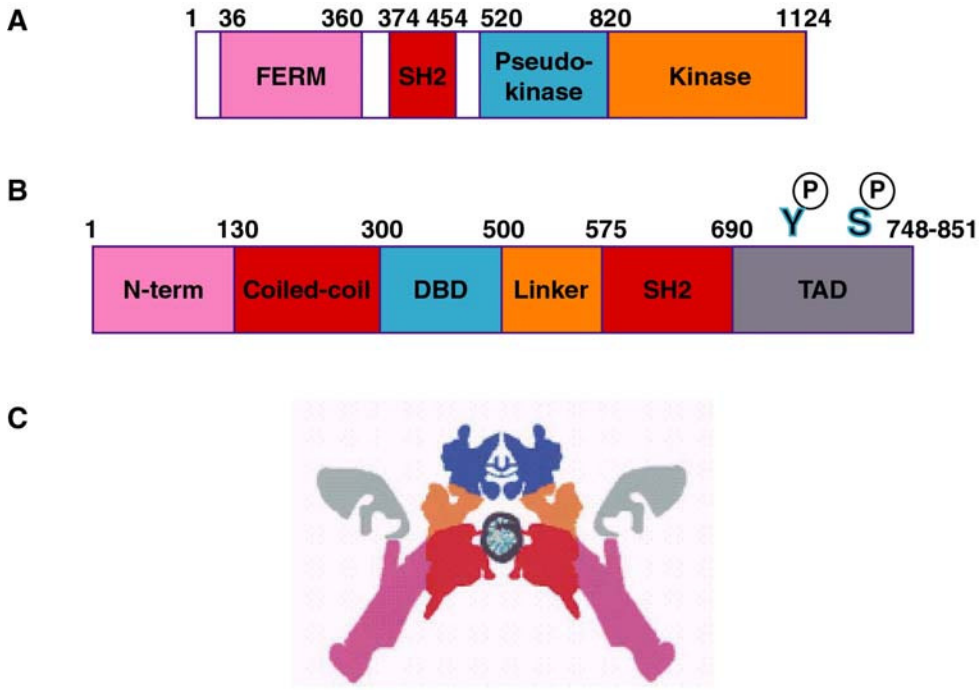


Figure 2. Structure of Jaks and Stats. (A) Schematic of Janus Kinase Structure. The FERM domain mediates receptor interactions. Both the FERM and pseudokinase domains regulate catalytic activity (FERM: Band four point one, ezrin, radixin and moesin homology domain).

(B) Structure of Stat proteins. Stats have amino-terminal, coiled-coil, DNA binding (DBD), linker, SH2, and transcriptional activation (TAD) domains. **(C)** The structure of a polypeptide lacking the amino terminal domain and the TAD has been solved and is shown. The placement is conjectural. (Adopted modified from O'Shea *et al.* Cell 2002. April, 109 S121-31.)

1.1.2. Biology of Stats There are seven known mammalian Stat proteins, Stat1, 2, 3, 4, 5a, 5b and 6 [8]. Figure 2B is a schematic diagram of the structural domains of Stats. Stats consist of an N-terminal domain (residues ~ 1-300), coiled coil domain (residues ~ 130-300), DNA binding domain (residues ~ 300-500), linker domain (residues ~ 500-575), SH2 domain (residues ~ 575-690), and transactivation domain (residues ~ 690-850). Figure 2C represents the three-dimensional structure of Stats lacking the N-terminal and transactivation domain bound to DNA.

Stat5a and Stat5b are products of two distinct genes with 96% homology. Stat5 homo- or heterodimers are activated by a number of growth factors including prolactin, growth hormone, erythropoietin, thrombopoietin, granulocyte-macrophage colony stimulating factor, and IL2, IL3, IL5, IL7, IL9, and IL15 [8]. However, their unique role in immune

function was revealed in mice deficient of Stat5a and 5b genes. Stat5a deficient mice show defective lobulo-alveolar development and milk production in the mammary glands of female mice [9]. Stat5b deficient mice are growth retarded similar to abnormalities found in Laron type dwarfism, a disease caused by growth hormone receptor defects [10]. Mice with a deficiency of both Stat5a and 5b genes are immune suppressed with the same abnormalities as seen in $Stat5a^{-/-}$ and $5b^{-/-}$ mice alone. T cells were unable to proliferate in response to T cell ligation in the presence or absence of IL2 [1]. T and B cell development were normal, but T cells derived from Stat5a/b double [1]. In addition, female mice were infertile due to the defects in development of the corpus luteum. A number of studies have also implicated the role of Stat5a/b in erythropoietin signaling and red blood cell development [11, 12].

In summary, the known physiological role of Stat5a/b is to mediate growth in response to growth hormone, proliferation of peripheral T lymphocytes in response to IL2, and lactation and fertility in female mice.

For IL2, binding of ligand to the heterotrimeric receptor complex recruits Jak1 and Jak3, which become activated by cross-phosphorylating their catalytic domains following receptor oligomerization. Jaks phosphorylate Stat5a/b recruited to the receptor on a tyrosine near their carboxyl terminal. Phosphorylation of Stat5 promotes their dissociation from the receptor chain, homo- or heterodimerization and nuclear transport. Binding of Stat5a/b to the consensus oligonucleotide sequence TTCC(C/G)GGAA, also known as GAS (Interferon γ Activated Sequence), initiates transcription of growth-related genes [13], IL2Rα [14], pim-1 [15], and cell cycle genes [1].

1.2. Syk and Lck Pathways

Syk is a tyrosine kinase that becomes activated in response to IL2 [16] (Figure 1). Its activation was shown to mediate transcription of genes that control cell proliferation. Syk-/- mice have a severe defect in B cell development, however T cells responded normally to IL2, suggesting that Syk activation is not essential for IL2-induced T cell proliferation [17].

Similarly, the Src tyrosine kinase family member Lck is also activated in response to IL2 and αCD3 stimulation of T cells. In TCR signaling, Lck plays a significant role in the phosphorylation of TCR chains and the activation of signaling molecules in the TCR activation pathways. Lck can be recruited to the IL2Rβ chain and becomes activated in response to IL2 [18] (Figure 1). Studies on Lck-/- mice demonstrated that Lck deficiency was associated with a defect in T cell development, however, T cells exhibited normal proliferation in response to IL2 [19].

1.3. PI3K/mTor (mammalian target of rapamycin) pathway

Phosphotidylinositol 3-kinase (PI3K) is a lipid kinase, which catalyzes phosphorylation of phosphatidyl-inositol (PI) to generate secondary messengers such as PI-3P, PI- 3, 4-bisphosphate (PIP2), and PI- 3, 4, 5-triphosphate (PIP3). IL2 signaling results in the phosphorylation and activation of PI3K [20]. PI3K signaling initiates activation of transcription factors such as E2F and a serine/threonine kinase known as $p70^{S6K}$ [21, 22]. E2F regulates the transcription of Cyclin E (a cyclin involved in G1 to S transition), and $p70^{S6K}$ activates S6, which activates 40S ribosomal protein essential for the synthesis and elongation of many ribosomal proteins. It is also believed that PI3K is involved in activation of mTor (mammalian Target of Rapamycin), a lipid kinase, which phosphorylates and represses 4EBP1 (Initiation Factor 4E Binding Protein), allowing for the release and activation of eIF4E (eukaryotic Initiation factor 4E), an initiation factor involved in mRNA capping [23]. PI3K signaling has also been implicated in cell survival. The product of PI3K activation (PI-3, 4, 5-triphosphate) recruits the serine/threonine protein kinase B (PKB), which phosphorylates Bad to prevent apoptosis [24]. These studies suggest PI3K plays an important role in T cell proliferation and survival via IL2.

1.4. Shc pathway (Mapk pathway)

Shc is an adaptor protein containing a phosphotyrosine binding SH2 domain [25]. Phosphorylation of IL2Rβ chain results in the recruitment and binding of Shc (Figure 1). Shc binding to the receptor leads to the recruitment of Grb2, another adaptor protein, and the nucleotide exchange factor, Son of Sevenless (SOS). SOS facilitates exchange of GTP for GDP on Ras and results in the activation of the classical Ras-Raf-Mapk pathway [26].

Receptor mutation studies have shown that the Mapk pathway promotes the proliferation and survival of T cells [27, 28]. However, it is not clear whether their role is essential and/or redundant with other pathways.

1.5. Negative Regulators of IL2 signaling

A number of negative regulatory proteins exist within the IL2 signaling pathway. These include proteases such as calpain, protein phosphatases (SHP-2, CD45), PIAS (Protein Inhibitor of Activated Stats), and CIS (Cytokine Induced SH-2 Containing Proteins) or SOCS (Suppressor of Cytokine Signaling) family (Figure 1).

Calpain is a calcium dependent cysteine protease, which was found to interact with the γc receptor chain and therefore downregulate the cytokine-mediated proliferation of murine thymocytes driven by T cell growth factors [29].

There are five members of the Protein Inhibitor of Activated Stats (PIAS) family: PIAS1, 3, x_a, x_b, and y. Only PIAS1 and PIAS3 have been shown to inhibit the DNA binding activity of activated Stat1 and 3, respectively [30, 31].

Another important negative regulator of IL2 signaling is the CIS (cytokine-inducible SH2-containing protein) family of proteins, also referred to as the SOCS (suppressors of cytokine signaling) or SSI (STAT-induced STAT inhibitor) [32]. Among these proteins, CIS-1 and SOCS-1 have been shown to inhibit IL2 mediated signal transduction [33, 34].

Protein phosphatases have also been shown to dephosphorylate and inactivate cytokine signaling [35]. SHP-1 and SHP-2 are SH2-containing tyrosine phosphatases shown to be implicated in termination of Jak/Stat pathway by dephosphorylation of the critical tyrosines within the activation loop of the Janus kinases [36, 37]. CD45, a transmembrane phosphatase, has also been demonstrated to down-regulate cytokine mediated signal transduction via dephosphorylating Jak kinases [38].

2. Potential Targets to Control Abnormal T Cell Function

Signal 1 (engagement of antigen by T Cell Receptor) and 2 (costimulatory interaction between B7-CD28) are critical for the synthesis and secretion of IL2, which, in concert

with other T cell growth factors (TCGFs) such as IL4, 7, 9, 13, and 15, deliver signal 3 through cytokine receptors, a necessary step for driving the clonal expansion of T cells [8]. These cytokine receptors share a common gamma chain that, when combined with a private α chain for each cytokine, delivers intracellular signals by Jak1 and Jak3 and that activates Stat1, Stat3, Stat5a/b, and Stat6 [2, 8, 39-42]. Current clinical immunosuppressive regimens are dominated by CaN inhibitors (cyclosporine [CsA] or FK50612) that block T cell progression through the early G1 stage of the cell cycle [43, 44]. However, ubiquitous expression of CaN in many different tissues contributes to several adverse side effects, including nephrotoxicity and neurotoxicity [45, 46].

Inhibition of molecular targets unique to lymphocytes, particularly those activated by TCGFs, would provide a novel and selective means to block T cell function and allograft rejection. In particular, identification of Jak3 antagonists would result in the inhibition of an entire family of TCGF-dependent pathways. Indeed, Jak3 (primarily expressed in T, B, and natural killer [NK] cells) is activated through the γc and plays a critical role in T cell development and function [47, 48]. In humans or mice, genetic inactivation of either the γc or receptor-associated Jak3 is manifested as severe combined immunodeficiency disease (SCID) [2-4, 40].

Additionally, earlier work showed that AG-490, a tyrphostin family member can inhibit Jak3 autokinase activity in PHA-activated human T cells, Stat5a/b activation, and subsequent T cell proliferation and allograft rejection [49, 50].

Moreover, another Jak3 inhibitor, PNU156804; an analogue of undecylprodigiosin, was found to block IL2-induced T cell proliferation by inhibiting the activation of NF-κB and AP-1 transcription factors, the expression of cyclins D2 and E, the cyclin dependent kinases 2 and 4, and the hyperphosphorylation of the retinoblastoma protein [51]. In contrast, IL2 receptor (IL2R) messenger RNA (mRNA) expression of α and γ chains was not affected by PNU156804 [51]. However, the molecular target for PNU156804 was not identified in this earlier study.

3. Aberrant Stat Activation in Neoplastic Transformation

The evidence that Stat proteins control normal mitogenic responses raised the possibility that their deregulated activation can directly contribute to cellular transformation. Aberrant receptor activation or protein tyrosine kinase activity can induce constitutive Stat signaling in oncogenesis. The first genetic evidence implicating aberrant Stat activation in the development of neoplasias was derived from studies with a lethal gain-of-function mutation of *Drosophila* Jak homolog, which results in hyperactive Jak kinase activity and causes leukemia-like defects [52, 53]. Dominant suppressors of this phenotype map to loss-of-function mutations in the *Drosophila* homolog of a mammalian Stat gene [54, 55]. Thus, these studies suggested that deregulated activity of these molecules can directly lead to the formation of hematopoietic malignancies.

Stat3, first cloned as Acute Phase Response Factor (APRF) [56], is ubiquitously expressed and activated by many ligands including IL6, EGF, PDGF, Oncostatin M, and LIF. In some instances Jaks, tyrosine kinase containing growth factor receptors, and non-receptor tyrosine kinases such as c-Src may serve as activators. Targeted deletion of Stat3 is embryonically lethal in murine models [57] while tissue-specific targeting of Stat3 in T cells yields impaired response to IL6 [58]. Stat3 has been found constitutively activated in various tumor cell lines and accompanied by upregulation of various genes (e.g. Cyclin D1, c-Myc, Bcl-x) [59] that control cell growth and survival. These cell lines have been derived from various tissues including breast, hematopoietic, brain and prostate in origin [60-63]. In human cancers dysregulation of Stat3 signaling can result from hyperactive ligands or their receptors, constitutive receptor-ligand complexes, aberrant functional properties of tyrosine kinases (such as c-Src) and tumor viruses, as well as putative downstream effectors of Stat3 [61, 64-66]. Recent models support this hypothesis since enforced expression of a constitutively active disulfide-linked Stat3 homodimer is able to drive tumor formation in nude mice [59]. Thus, defining mechanisms to uncouple constitutively active Stat3, which acts to drive aberrant growth and promote cell survival, should be therapeutically beneficial to treat several types of diseases.

4. Serine Phosphorylation of Stats

Current models suggest that activation of Stats is mediated by two distinct kinases, a tyrosine kinase such as Jak and/or Src enzymes and one of several putative Stat serine kinases [67]. Only the dually phosphorylated Stat would be competent to disengage from the receptor, dimerize, nuclear translocate, bind select DNA sequences and associate with transcriptional molecules necessary for maximal gene transcription [68]. The Stat phosphoacceptor sites are represented by a highly conserved tyrosine residue, while the serine is typically located within one or more proline rich motifs within the transactivation domain [69]. For Stats 1, 3 and 4, this site has been mapped to S727 and is required for full gene transcription [67, 70-74]. In T cells, non-cytokine dependent signals can also regulate this site with studies showing that TCR engagement can drive S727 phosphorylation of Stat1 and Stat3 [71, 72]. CD28 ligation also increased S727 phosphorylation within Stat1 [75] whereas crosslinking of FCgammaRIIa stimulated Stat3 S727 phosphorylation in B cells [76]. Several serine kinases have been demonstrated to phosphorylate the PMSP-motif, a consensus Mapk phosphorylation sequence [77] that has been further substantiated by co-precipitation of Erk1/2 with Stats [73, 78] and inhibited by Mek1/2 poisons [72, 73, 79]. Non-cell surface receptor activation of Erk1/2 by phorbol esters that utilize PKC-Raf intermediates also increased Stat3 serine phosphorylation [72, 80]. However new reports have identified PI3K [75, 76] and mTor [81] as candidate Stat serine kinases while p38 and Jnk [79, 82, 83] have been mostly discounted.

Unlike Stat1, 3 and 4, less is known about the Stat5a/b serine kinase regulation. While tyrosine phosphorylation sites of human Stat5a/b has been mapped to Y699/701 [84] respectively, the serine phosphorylation sites and putative kinase is less clear. Increasing evidence suggests this site lies within the transactivation domain and is represented by a PSP-motif that lacks the invariant methionine residue, and thus a weak Mapk target [77]. Earlier work by Yamashita *et al.* employed alanine scanning mutagenesis, reconstitution assays and phosphoamino acid analyses of prolactin treated COS-7 cells to identify these phosphoacceptor sites in murine Stat5a S725 (human S726) and Stat5b S730 (human S731) [85]. Supportive evidence in the form of functionally relevant pro-B-cell and T cell lines lends credence to these findings since delivery of carboxyl terminal deleted Stat5

variants lacking these serine residues inhibits cytokine-driven cell proliferation and cell cycle gene expression [13, 86]. Recently published findings by Park *et al.* suggest these Stat5a/b serine sites are critical for maximal growth hormone regulated gene transcription [87]. Nonetheless, given the central role of Stat5 in T cell activity and cancer, identification of the kinase and its regulation makes it a critical target for controlling various diseases.

5. Antisense oligonucleotides: molecular tools to modulate gene expression

Current difficulties with non-specific inhibitors to block T cell signaling pathways to determine their function is a challenge. However, employing antisense oligonucleotides may aid in this dilemma.

The notion that antisense oligonucleotides could be used to modulate gene expression was first proposed by Zamecnik and Stephenson in 1978 [88]. The employment of this strategy initially generated a lot of controversy due to numerous technical difficulties, which may have been caused by unreliable introduction of antisense oligonucleotides to cells and lack of screening for direct antisense effects on target mRNA and subsequent specific reduction of target protein.

Antisense oligonucleotides are designed to modulate the transfer of information from the gene to the protein by interfering with the function of mRNA or pre-mRNA. Due to the fact that protein-encoding RNA undergoes a wide range of essential processing steps prior to the completion of a protein synthetic event, it is theoretically possible to modulate RNA function with antisense by interfering with any one of these processing reactions. For example, hybridization of an oligonucleotide to specific RNA sequences to prevent the binding (steric inhibition) of important regulatory proteins may result in the modulation of RNA splicing, polyadenylation, translation, or degradation. Activation of a cellular enzyme called RNase H results in the degradation of the RNA strand of the RNA-DNA double helix, thus decreasing the amount of the protein of interest in the cell (Figure 3).

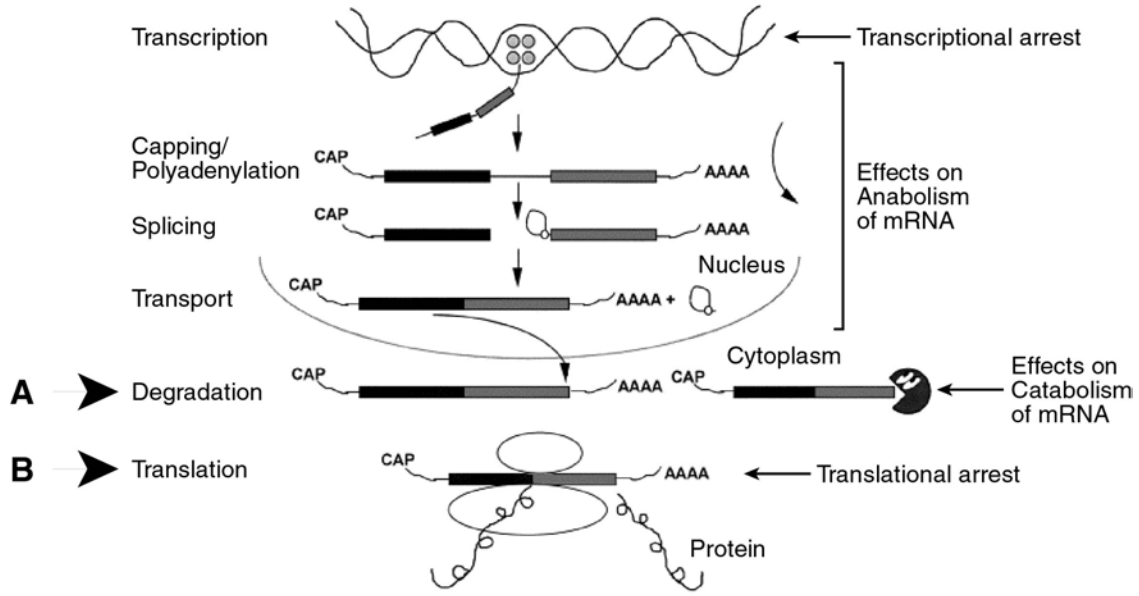


Figure 3. RNA processing. Each step shown in the pathway is a composite of numerous steps and is theoretically amenable to intervention with oligonucleotides. A: RNase H mediated antisense effect B: Steric inhibition (non RNase H mediated, translational arrest). (Adopted modified from Crooke S. T. BBA 1489(1) 31-43)

A very attractive feature of antisense oligonucleotides is the *potential for specificity*. Many genes encoding critical regulatory molecules occur as multigene families of closely related members. Further, many of these families are characterized by sharing highly conserved functional domains among family members. Antisense oligonucleotides target mRNA (Figure 3) and are able to take advantage of subtle differences in the genetic code of closely related family members. Secondly, antisense oligonucleotides can be used to *inhibit the expression of a wide variety of gene products*, including receptors, ligands, enzymes, etc.

5.1. Practical considerations in the use of antisense oligonucleotides

5.1.1. Identification of optimal target sequences

The design of antisense inhibitors is more complicated in practice than in theory, due to the finding that not only the binding affinity of the antisense to the mRNA molecule but also the accessibility of the target RNA influences the inhibitory effect. The

inaccessibility of the target RNA to the oligonucleotide is likely the result of the secondary or tertiary structure in the RNA and/or proteins bound to the RNA. This limitation can be overcome by screening multiple oligonucleotides targeted against the mRNA of the protein of interest [89-92].

5.1.2. Antisense oligonucleotide uptake

In vitro cellular uptake and subcellular distribution of oligonucleotides have been demonstrated to be driven by chemical class effects and are largely unaffected by sequence-dependent effects. In general addition of oligonucleotides directly to the culture media does not result in efficient biological activity. To facilitate oligonucleotide uptake in vitro, a number of methods have been tried such as: cationic lipids [93] and liposomes, peptides [94], polycations [95], conjugation with cholesterol [96], aggregation with cell surface ligands [97] and electroporation [98]. Cationic lipids are the most widely used method for most adherent cells; however, they have proven to be less effective for non-adherent leukocyte derived cells or cell lines. It is necessary to optimize delivery of antisense oligonucleotides in the cell system of interest.

5.1.3. Antisense oligonucleotide stability

Antisense oligonucleotides are chemically synthesized which allows the alteration of the properties of the molecule (stability, pharmacokinetics, potency and toxicity) to optimize for a specific application. In cell culture experiments the nuclease resistant phosphorothioate modification is the most widely utilized and often referred to as "1st generation antisense oligonucleotide" [99-101]. Other, more recently characterized modifications such as 2'-*O*-methoxyethyl or morpholino are called "2nd generation antisense oligonucleotides" [102]. Figure 4 is a summary of chemical modifications of antisense oligonucleotides.

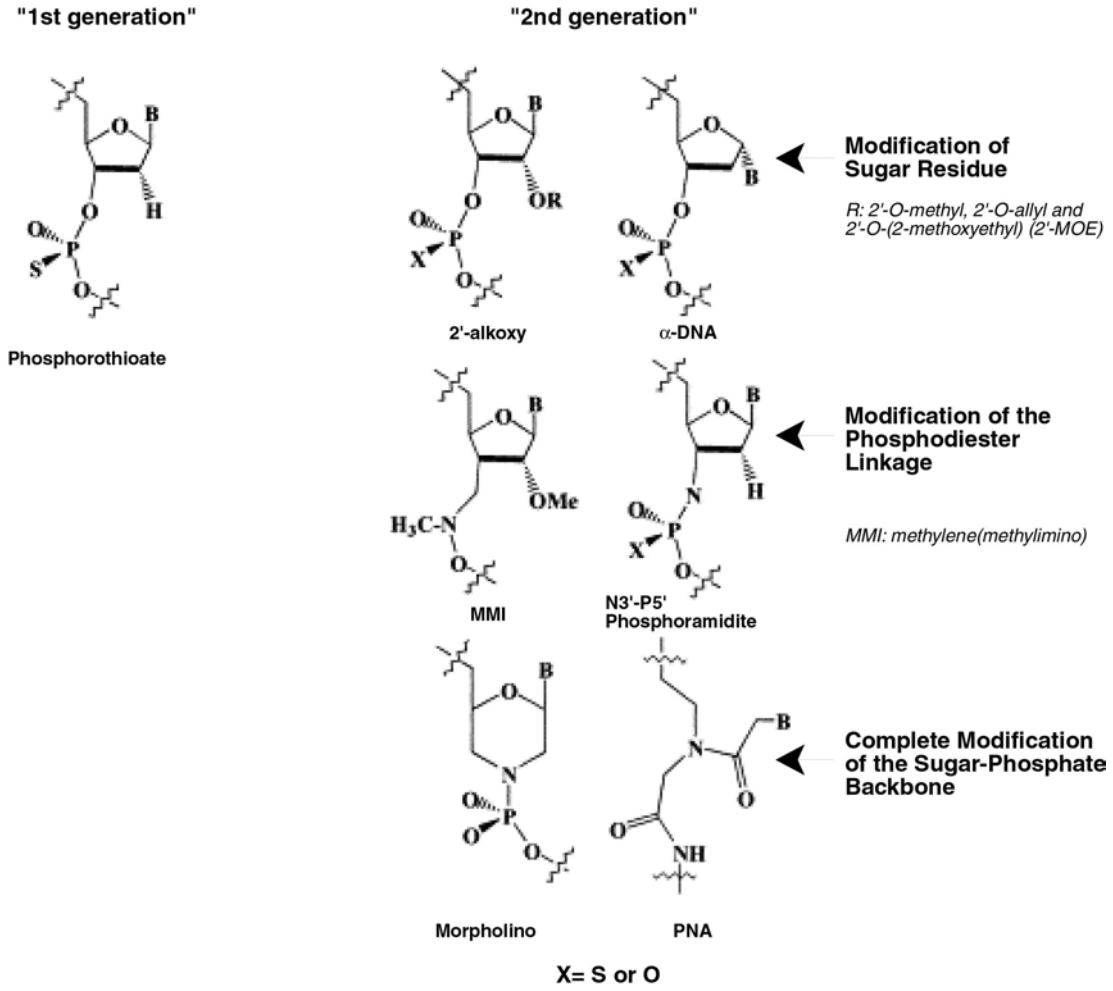


Figure 4. Chemical modifications of antisense oligonucleotides. First generation antisense oligonucleotides are phosphorothioate modifications, which supports RNase H activity. Second generation oligonucleotide modifications that do not support RNase H activity may be generally categorized as follows: modification of the sugar residue (I), modification of the phosphodiester linkage (II), or complete modification of the sugar phosphate backbone (III). (Adopted modified from Baker *et al.* BBA 1489(1) 3-18)

5.2. Factors influencing antisense activity

5.2.1. Affinity results from hybridization interactions and is directly proportional with ionic strength. Affinity increases with the length of the oligonucleotide-receptor complex, as well as the sequence in the duplex. Several factors contribute to the differences between theoretical and realized affinities, for example secondary and tertiary

structures of the target RNA, RNA-protein interactions inside the cell, which makes the *in vivo* situation even more complicated [103-105].

5.2.2. Specificity derives from the selectivity of Watson-Crick base pairing. Any sequence of 17 residues is expected to occur statistically only once at the genomic level. Assuming a random distribution of sequences in RNA, any sequence of 13 residues is expected to occur once in the cellular RNA population. Considering the non-random nature of mammalian RNA sequence, an 11-mer oligonucleotide or perhaps smaller could identify and bind to a unique sequence [106]. In practice, 15-25-mer antisense oligonucleotides are the most widely utilized.

5.2.3. Toxicity results from the non-specific interactions between antisense oligonucleotides and cellular components. Chemical classes of oligonucleotides differ in their tendency to interact with various non-nucleic acid targets. For example, phosphorothioates tend to bind to a wide range of proteins with relatively low affinity [107]. *In vitro* and *in vivo* toxicological studies have shown that these interactions probably reduce the therapeutic index of phosphorothioates less than it was expected [108]. This might be due to the fact that the phosphorothioates bind with very low affinity to a large number of proteins and their potential toxic effects are consequently buffered. Many of these effects have been described and can be avoided by proper design of the antisense inhibitor or controlled for with appropriate mismatched and scrambled controls [109, 110].

5.2.4. Activation of RNase H RNase H is a ubiquitous enzyme that degrades the RNA strand of an RNA-DNA duplex, has been identified in diverse organisms from viruses to human cells. At least two classes of RNase H have been identified in eukaryotic cells, and data exist suggesting the presence of multiple isozymes. Multiple enzymes with RNase H activity have been observed in prokaryotes. Although RNase H is involved in DNA replication, it may play other roles in the cells and is found in the cytoplasm as well as the nucleus [111].

The precise recognition elements for RNase H are not known. It has been shown that oligonucleotides with DNA-like properties as short as tetramers can activate RNase H

[112]. Sugar modifications that result in RNA-like oligonucleotides, e.g. 2'-fluoro or 2'-*O*-methyl do not appear to serve as a substrate for RNase H [113, 114]. Alterations in the orientation of the sugar to the base can also affect RNase H activation, since α-oligonucleotides are unable to serve as substrates for RNase H [115]. Additionally, backbone modifications influence the ability of oligonucleotides to activate RNase H. Methylphosphonates do not serve as RNase H substrates [116], while phosphorothioates are excellent substrates [117]. Chimeric oligonucleotides, for example oligonucleotides comprised of wings of 2'-*O*-methyl sugars and methylphosphonate backbone and a 5-base gap of oligodeoxynucleotides can bind to their target RNA and activate RNase H [118]. Lastly, a single ribonucleotide in a sequence of deoxyribonucleotides was shown to be sufficient to serve as a substrate for RNase H when bound to its complementary deoxyoligonucleotide [119].

5.3. Biological activity of chemically modified antisense oligonucleotides

The most-studied of the oligodeoxynucleotides are the phosphorothioate analogs (PS-oligos), in which one of the non-bridging oxygen atoms in the phosphate backbone is replaced by a sulfur [99-101]. The mechanisms of action can be classified into three categories: (a) sequence-specific activity by binding to mRNA (antisense activity); (b) sequence-specific activity by interacting with other factors (non-antisense activity); and (c) non-sequence-specific activity. PS-oligos have a negatively charged backbone and are capable of supporting RNase-H activity similar to phosphodiester oligodeoxynucleotides, but PS-oligos have greater resistance to nuclease degradation than do phosphodiesters [99-101]. PS-oligos were initially used as inhibitors of HIV-1 replication, but the apparent mechanism differed depending on the experimental model system used [120-128]. It has also been shown in a number of studies that PS-oligos with CpG motifs have immune-stimulatory properties in rodents [129-134].

The nucleotide composition and nature of the nucleotide and internucleotide linkages alone, or in combination, dictate the biophysical, biochemical, and biological properties of oligonucleotides [135]. A number of oligonucleotide analogs display properties different from those of PS-oligos in terms of resistance to nucleases, affinity to target RNA, cellular

uptake and activation of RNase H [99-101]. For optimum activity (decrease the polyanionic nature and immune-stimulatory effects), antisense oligonucleotides having combination of various properties instead of only increased stability toward nucleases or high affinity to target RNA were generated and referred to as mixed-backbone oligonucleotides (MBO) [136] or “second generation” antisense oligonucleotides [102].

V. EXPERIMENTAL PROCEDURES

Cell culture and treatment: Freshly explanted human T-lymphocytes were purified and maintained with PHA (1 μg/ml) in RPMI-1640 medium containing 10% fetal calf serum, 2 mM L-glutamine and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively). The human YT and murine CTLL2 cell line was maintained in the above media in the absence of PHA with the latter line supplemented with 50 U/ml recombinant human IL2 (PeproTech). Approximately 1 x 10^8 cells were then stimulated with media or 100 nM recombinant human IL2 (Hoffmann-LaRoche, Nutley, NJ), IL7, IL9, IL15 (PeproTech, Rock Hill, NJ), 5μg/ml αCD3 antibody (PharMingen), or 1μg/ml phorbol 12-myristate 13-acetate (ethanol stock concentration 1 mg/ml; Sigma) at 37^{o}C at times indicated in the corresponding figure legends. Cell pellets were frozen at -70 ^{o}C until use.

Solubilization of membrane proteins and immunoprecipitation: Cells were solubilized in lysis buffer (10mM Tris, 5mM EDTA, 50mM NaCl, 1% Triton X-100; 10^8 cells/ml) and clarified by centrifugation with 12000g at 4^{o}C for 30min. For immunoprecipitation reactions, supernatants were incubated with 5μl/ml polyclonal rabbit antiserum raised against peptides derived from the extreme carboxyl termini of murine forms of Stat5a, Stat5b or Stat3. Site-specific anti-Stat5-phosphoserine antibodies were produced to corresponding phosphopeptide sequence DQAP[pS]PAVC. Blots were Western blotted with monoclonal mouse anti-phosphotyrosine or anti-phosphotyrosine Stat5a/b antibodies (Upstate Biotechnology Inc.), or monoclonal anti-Stat5 (Transduction Laboratories) at 1:1000. Immunoprecipitation of Raf isoforms was performed as described below. For serine kinase inhibitor experiments, cells were pre-incubated for 1 h with either Me_2SO as a mock control, or with varying concentrations of PD98059 (New England Biolabs Inc.), rapamycin and wortmannin (Calbiochem) and then lysed and immunoprecipitated as described in figure legends. For all samples, total protein was determined by BCA method (Pierce).

Antisense ODN treatment and viability assay: YT or CTLL2 cells were treated with selective antisense to each raf isoform (ISIS Pharmaceuticals Inc.). The phosphorothioate backbone was synthesized using phosphorothioate chemistry, and 2'-methoxyethyl

modification of the five terminal nucleotides (underlined) used to increase stability (underlined base) of the oligonucleotide sequences employed were as follows:

Human A-raf (ISIS 15489) 5'-CTAAGGCACAAGGCGGGCTG-3',

Mouse/human B-raf (ISIS15344) 5'-CTGCCTGGATGGGTGTTTTT-3',

Human C-raf (ISIS13650) 5'-TCCCGCCTGTGACATGCATT-3',

Mouse A-raf (ISIS 15493) 5'-CTAAGGCACAAGGCGGGCTG-3',

Mouse C-Raf (ISIS 15770) 5'-ATGCATTCTGCCCCCAAGGA-3'.

YT or CTLL-2 cells in exponential growth phase were re-suspended at 7.5 x 10^7/ml in ice-cold OPTI-MEM-I medium. A 400µl sample of these cells were placed in cuvettes with oligo-deoxynucleotides at varying concentrations ranging from 5-20 µM then electroporated at 220V and 960µF using a Bio-Rad Gene Pulser™. Viability was assessed by Trypan-Blue dye exclusion using hemacytometer or MTS assay (described later).

For the Stat3 antisense the same modifications were synthesized, and the oligonucleotide sequences employed were as described previously [137].

Fully phosphorothioate human IL2Rγ antisense: 5'- GTAATGATGGCTTCAAC-3' and its scrambled control: 5'-ATATTACGCGATAGGTC-3'.

Raf in vitro kinase assays: IL2 treated and untreated cells were lysed and normalized protein amount immunoprecipitated with A-, B- (Santa Cruz Biotechnology, Inc.) or C-Raf antibodies (BD Pharmingen) as described above. Immuno-complexes were washed with lysis buffer three times followed by washing with kinase buffer containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT. Kinase reaction was carried out in the presence of 50 µM ATP, and 10 µCi [^{32}P]-ATP (NEN DuPont) for 10 min at 37°C. Samples were washed three times with lysis buffer, boiled for 5 min and subjected to 8% SDS-PAGE under reducing conditions. For substrate assay, 0.25 µg/µl kinase-dead MEK1-GST was used for each sample. In both experiments, reactions were terminated by adding 10 µl 4x SDS-sample buffer, separated by SDS-PAGE, transferred to PVDF membrane and exposed to X-ray film (X-Omat, Kodak) at -70 °C.

Electrophoretic mobility shift assay (EMSA): Nuclear extracts were prepared from IL2, IL7, IL9, or IL15 stimulated T cells (described above) or from non-stimulated or IL2 stimulated YT cells (described in figure legends) then pelleted by centrifugation (20,000 x g

for 1 min at 4 °C) and subsequently washed in five volumes of 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 25 mM NaF, 100 mM PMSF, 5 μg/ml aprotinin, 1 μg/ml pepstatin A and 2 μg/ml leupeptin, centrifuged, then lysed in the same buffer supplemented with 1% NP-40 and incubated for 20 min on ice. The nuclei-containing pellet was resuspended in equal volumes of low salt buffer (10 mM HEPES, 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.5 mM DTT, 0.2 mM EDTA and protease inhibitors) and high salt buffer (supplemented with 800 mM KCl). The nuclear extraction was centrifuged at 4°C for 10 min and the supernatants were saved as nuclear protein extracts and stored at -70°C. 5 μg nuclear extracted proteins from each sample were incubated in 15 μl of binding cocktail (50 mM Tris-Cl, pH 7.4, 25 mM $MgCl_2$, 0.5 mM DTT, 50% glycerol) at 4 ^oC for 2 h. For supershift assays, the nuclear extracts were pre-incubated with 1 μg of either normal rabbit serum or antisera specific to Stat5 or Stat3 as indicated in corresponding legend at 4 ^oC for 20 min. Samples were then incubated with [^{32}P] endlabeled oligonucleotide corresponding to the β-casein gene sequence (5'-AGATTTCTAGGAATTCAATCC-3') or to the c-*fos* promoter *sis*-inducible element (SIE) m67 (5'-TGTCGACATTTCCCGTAAATC-3') for 15 min at room temperature and subjected to gel electrophoresis.

Proliferation Assay: Quiescent cells (50 x 10^3/well) were plated in flat-bottom 96-well microtiter plates in 200 μl of growth medium (described above), employing 1% FCS in the presence or absence of IL2 (1 nM). Cells were treated with PD98059, rapamycin, wortmannin (for 16 h) or A-, B- and C-raf antisense oligonucleotides (for 40 hrs) and pulsed for the remaining 4 h of the assay with [^3H]-thymidine (0.5 μCi/ 200 μl), and harvested onto glassfiber filters. [^3H]-thymidine incorporation was analyzed by liquid scintillation counting.

MTS assay: Following electroporation, cells were plated in flat-bottom 96-well microtiter plates (2-4 x 10^4/ml, 200 μl/well, triplicates) and cultured for time as indicated in figure legends in a CO_2 incubator. 20 μl of MTS reagent (CellTiter, Promega) was added to each well, incubated for another 2 hrs and optical density (OD) was determined at 490 and 630 nm. OD is proportional to the number of viable cells.

Annexin V- Propidium-Iodide double staining: 1-2 x 10^6 cells were centrifuged for 5 min at room temperature with 800 rpm, washed with PBS and re-suspended in binding buffer

containing Annexin V and propidium-iodide (Annexin-V-FLUOS Staining Kit, Roche), incubated for 30 min at room temperature and analyzed with flow cytometer (Beckman Coulter, Inc.).

Cell Cycle Analysis: For cell cycle analysis 1-2 x 10^6 cells were washed in PBS and fixed in 70% ethanol for at least overnight at 4 °C. After a washing with PBS, cells were stained with 50 μg/ml propidium-iodide in the presence of 5 μg/ml Rnase A (Ambion, Austin, TX) and analyzed for DNA content by FACS.

TUNEL (TdT-mediated dUTP-X Nick End Labeling): Cells were deposited on slides by standard cytospin procedure, fixed in 4% paraformaldehyde and incubated with the reaction mixture (TdT end labeling cocktail, containing fluorescein-12-dUTP as a substrate). TdT catalyzes the addition of fluorescein-dUTP at free 3'-OH groups in single- and double-stranded DNA. Slides were mounted in Vectashield with propidium-iodide, in order to prevent rapid loss of fluorescence and counterstain DNA for quantification. (Visualization by fluorescence microscopy.)

Statistical analyses: Differences in proliferation between controls and various treatment groups were tested by One Way ANOVA test with Tukey's posthoc analysis. Student's t-test was applied for pairwise comparison of treatments. All statistical analyses were carried out using SigmaStat (Jandel, San Rafael, CA) software systems. A value of $P<0.05$ was considered statistically significant (*).

VI. RESULTS

CHAPTER 1

Study the effects of selective inhibition of γc receptor and Jak3 in lymphoid cells

Introduction

IL2 receptor gamma chain (γc) is indispensable for Jak3 activation and signaling, and is a shared subunit of receptor complexes utilized by several T cell growth factors (TCGFs), including IL2, 4, 7, 9, 13, 15, 21. Jak3, which is primarily expressed in lymphocytes, binds to the γc and is necessary for T cell development and function [40, 47, 48, 138, 139]. The importance of Jak3/γc association and signaling pathway has been demonstrated by gene targeting in mice and by the identification of mutations in the γc and Jak3 genes of humans with severe combined immunodeficiency (SCID) [2-4, 40]. Since the abnormalities of such deficiencies are limited to the immune system, we focused our attention on the inhibition of IL2 signaling at the level of γc and Jak3 as potential immunosuppressive therapy of T cell mediated disorders. The exact role of γc in IL2 signaling is still not fully understood. Expression of a carboxyl terminal truncated form of the γc subunit in γc knockout mice restored T cell development through the upregulation of Bcl-2, but T cell function was abnormal [140].

These defects could be developmentally regulated. However, for a therapeutic viewpoint, the effect of blocking γc/Jak3 in mature lymphocytes is more relevant.

To do this, first we utilized selective phosphorothioate antisense oligonucleotides directed to γc and present its effects on the proliferation and survival of a human lymphoid cell line.

Next, we provide evidence that PNU156804, which is an analogue of undecylprodigiosin and was found to block T cell proliferation [51], selectively blocks downstream signaling pathways of Jak3 such as the Mapk cascade and Stat5.

Results

γc antisense decreases protein expression in YT cells. To investigate the role of γc in IL2- mediated signals, we chose to use phosphorothioate antisense oligonucleotides to specifically disrupt the expression of γc chain in YT cells (Figure 5). Cells were electroporated in the presence of 15μM antisense oligo (As γc), scrambled control (Scr

Ctrl) or with no oligo as described in the Experimental Procedures and cultured for 72 hrs. Following lysis, γc was immunoprecipitated from 1mg protein per each sample, and Western blotted with anti-γc antibody (Panel A, upper insert). As shown in lane c, γc protein was reduced while GAPDH was unchanged. Bcl-2 expression has been shown to be linked to γc signaling [141]; therefore we sought to determine whether decreased expression of γ_C affected Bcl-2 protein level. Total cell lysate (20 μg) of was separated by SDS-PAGE and blotted with anti-Bcl-2 and anti-GAPDH antibodies (panel A, middle and lower inserts, respectively). Panel B shows the quantitation of the blots. The density of γc was normalized against GAPDH and plotted on the y-axis as arbitrary units. Antisense treatment decreased the γc protein level 40% normalized to GAPDH suggesting that the antisense inhibits γc expression. Bcl-2 protein level was weakly reduced by both the γc antisense and the scrambled control oligos, indicating a mild nonspecific effect of the treatment. These data suggest that Bcl-2 expression is not mediated by the signaling via γc in these cells for the time point tested.

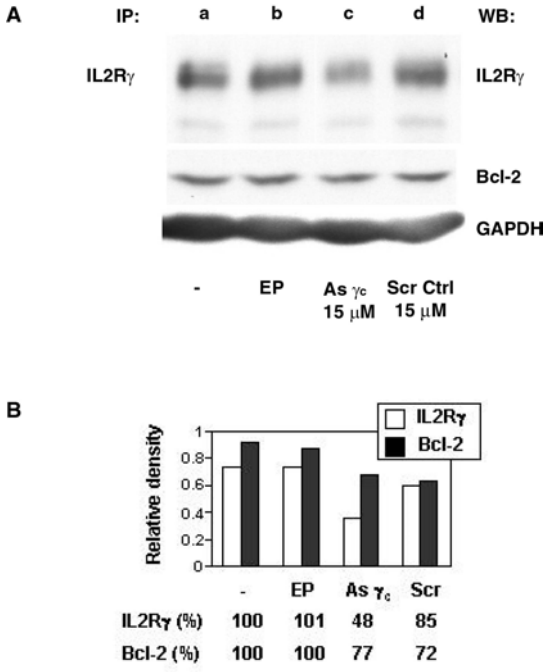


FIGURE 4. Inactivation of γc by antisense oligonucleotide inhibits protein expression. Cells were electroporated in the presence of 15μM antisense oligonucleotide (As γ_C) or scrambled control (Scr Ctrl), with no oligo (EP) or left untreated (-) as indicated. After 72 hrs, cells were lysed, immune complexes from equal amount of total cell lysates immunocaptured with α−γc antibody then subjected to SDS-PAGE and

blotted with α–γc. To assess Bcl2, 20 μg of the total lysates were resolved on 12 % SDS-PAGE and blotted with α–Bcl-2 antibody. Equal loading was confirmed by reprobing the blot with antibody against GAPDH. Densitometric analysis of the blots is shown on the right.

Diminished expression of the γc chain decreases YT cell viability. Since IL2 and the other cytokines sharing the γc play important role in the growth and survival of lymphoid cells [8], we wanted to determine the effect of reduced γc expression (Figures 6 and 7). Cells were electroporated in the presence of 10 and 15μM antisense γc, 15μM scrambled control, or with no oligonucleotide, then harvested at 72 hrs post-transfection, and viability was determined by MTS assay (Figure 6). The antisense treatment reduced the number of viable cells 43% compared to the scrambled oligo treated sample.

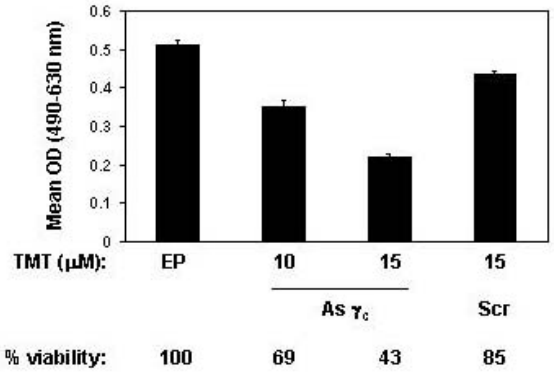
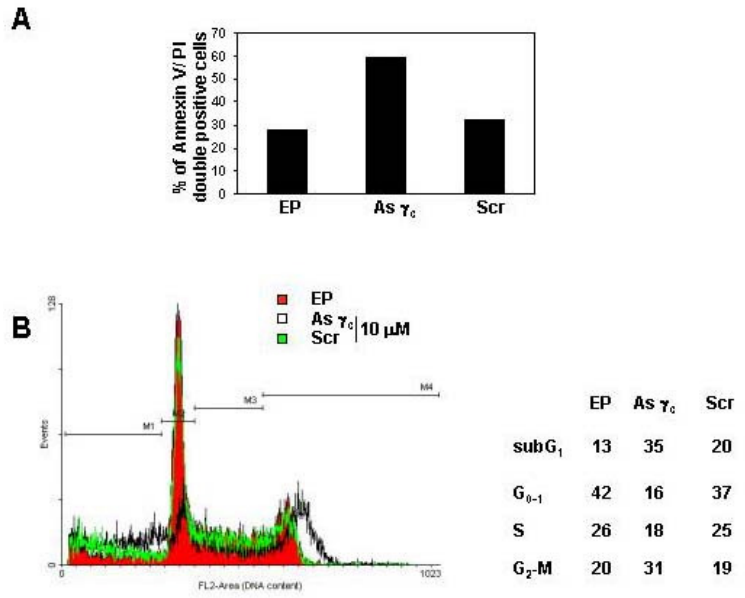


FIGURE 6. Antisense to γc decreases lymphoid cell viability. Cells were treated with antisense to γc as described above and 50 x 10^3 seeded into 96-well plates. Viability was determined at 72 hrs by MTS assay as described in Experimental Procedures.

Reduced expression of γc chain induces apoptosis and cell cycle arrest. The number of apoptotic cells was assessed by Annexin V and propidium-iodide (PI) double staining followed by FACS analysis (Figure 7A). Doubly stained cells represent the late apoptotic population, which was increased with 40% compared to the scrambled oligo treated samples. These data suggest that γc plays a crucial role in mediating lymphoid cell viability and survival.

In order to assess whether antisense treatment affects cell cycle progression, cells were electroporated without oligo (red), 10μM antisense γc (black) or scrambled oligo (green) and harvested 36 hours later, and analyzed for cell cycle progression by FACS. As shown on Figure 7B, antisense treatment reduced the number of cells in G_{0-1} (21%), increased $subG_1$ (15%) and G_2-M (12%) phases compared to the scrambled oligo treated sample, suggesting that suppressed expression of the γ_c induces cell cycle arrest in the G_2-M phase of the cell cycle.



	EP	As γ_c	Scr
$subG_1$	13	35	20
G_{0-1}	42	16	37
S	26	18	25
G_2-M	20	31	19

FIGURE 7. (A) γc antisense treatment induces apoptotic cell death. Cells were electroporated in the presence of 15μM antisense oligonucleotide (As γc), scrambled control (Scr Ctrl) or without oligo (EP). At 72 hours post EP, $1x10^6$ cells were washed twice with PBS and stained with Annexin-V-Fluorescein and Propidium-Iodide (PI) as described in Experimental Procedures. Cells were then analyzed by fluorescence-activated cell sorting (FACS). Double-positive cells represent a population in late apoptotic phase. **(B) γc antisense treatment causes cell cycle arrest.** Cells were electroporated in the presence of 10 μM antisense oligonucleotide (As γc) or scrambled control (Scr Ctrl) or with no oligo (EP). After electroporation (36 h), cells were fixed in 70% cold ethanol overnight, stained with PI working solution (50 μg/ml PI, 20 μg/ml RNAse, 0.5% Tween 20 in PBS) and analyzed for cell cycle progression by flow cytometry.

The above results suggested γc might be critical for lymphoid cell survival. To show that the γc-associated Jak3 mediated signals might be responsible for dictating this cellular

process; we next identified a Jak3 inhibitor to support the aforementioned model. Earlier work by our lab showed that PNU156804 blocks T cell proliferation by disrupting Jak3 activity and downstream signaling events [142].

PNU156804 disrupts IL2-mediated Stat5a/b Tyr/Ser phosphorylation. Given that both Stat5a and Stat5b are downstream of Jak3 and that T cells from Stat5a/b gene-deficient mice failed to proliferate in response to IL2 stimulation [8], we examined whether PNU156804 inhibits IL2-induced activation of Stat5a/b. For this assay, PHA-activated quiescent human T cells were treated with 20 μM PNU156804 or inactive control PNU159744 for 16 hours, followed by IL2 stimulation. PNU156804 blocked Stat5a (Figure 8A) and Stat5b (Figure 8B) tyrosine phosphorylation. In addition to tyrosine phosphorylation, Stat5a/b transcription factors are (like other Stats) also serine phosphorylated [8]. We previously mapped a serine phosphorylation site in Stat5a (S726) that is conserved in Stat5b (S731) [85, 143, 144]. As shown in Figure 8, PNU156804 inhibited IL2-induced Stat5a/b serine kinase activity. In particular, neither site was inducibly serine phosphorylated in the presence of cytokine and PNU156804, as measured by phosphoserine-specific Stat5a/b pAb (middle panels). The same samples reblotted with anti-Stat5a/b mAb confirmed equivalent protein levels (panels A and B). These results suggest that PNU156804 inhibits Jak3 from mediating tyrosine and serine phosphorylation of Stat5. In contrast, the inactive PNU159744 analogue showed no effect on either tyrosine or serine kinase activity. Because Jak-regulated Stat tyrosine/serine phosphorylation is required for dimerization, nuclear translocation, and gene transcription [67], we conclude that IL2-Stat5a/b-mediated gene transcription critical for IL2-mediated cell cycle progression is one explanation for the loss of IL2-inducible T cell proliferation [144].

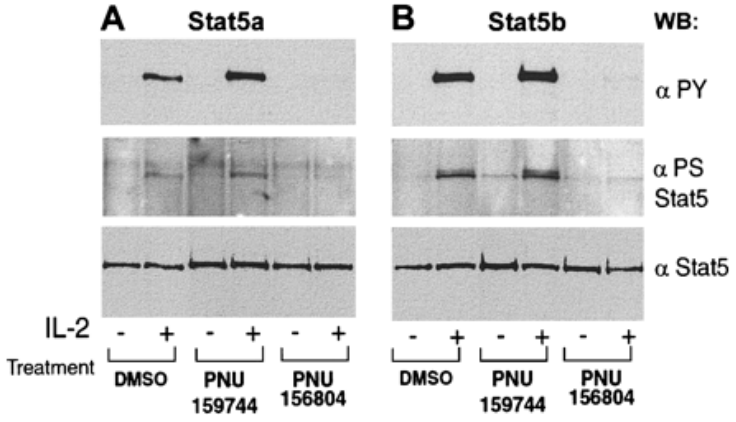


Figure 8. PNU156804 inhibits IL2-inducible Tyr phosphorylation of Stat5a/b in human T cells. PHA-activated human T cells were pretreated with DMSO, 10 μM in active analogue PNU159744, or 10 μM PNU156804 for 16 hours and then stimulated for 10 minutes with (+) or without (-) 100 nM IL2 for 10 minutes at 37°C. Cells were lysed and immunoprecipitated with anti-Stat5a (A) or anti-Stat5b (B) and were blotted with monoclonal antiphosphotyrosine Stat5 (Y701, upper panel), polyclonal phosphoserine Stat5 (S726-Stat5a or S731 Stat5b, middle panel), and reblotted with monoclonal pan-Stat5 (lower panel).

PNU156804 inhibits Erk1/2 Tyr/Thr phosphorylation. In addition to the Stat pathway, IL2 potently activates the Shc/Ras/Raf/MAPK cascade by the adapter protein SHC, which binds to Y338 of the IL2Rβ chain ultimately to potentiate T cell proliferation [28, 145]. To investigate whether PNU156804 disrupts this signaling pathway, PHA-activated T cells were treated with vehicle alone (Figure 9, lanes a-b) or with ascending PNU156804 concentrations. Total cell lysates separated on 10% SDS-PAGE were blotted with phospho-Erk1/2 antibody that recognizes activated T202 and Y204 sites on both enzymes. As shown in the representative experiment, Erk1 and Erk2 were completely inhibited at 10 μM PNU156804 (lane i). Two additional experiments confirmed the loss of active Erk1/2 proteins observed at 10 to 20 μM PNU156804 concentrations. Immunoblotting with a pan-Erk1/2 (indicated beneath phosphorylation blots) verified equivalent loading, although Erk2 was consistently detected. These data indicates that the inhibition of Jak3 by PNU156804 disrupts Mapk cascade activity in IL2-mediated signaling pathways.

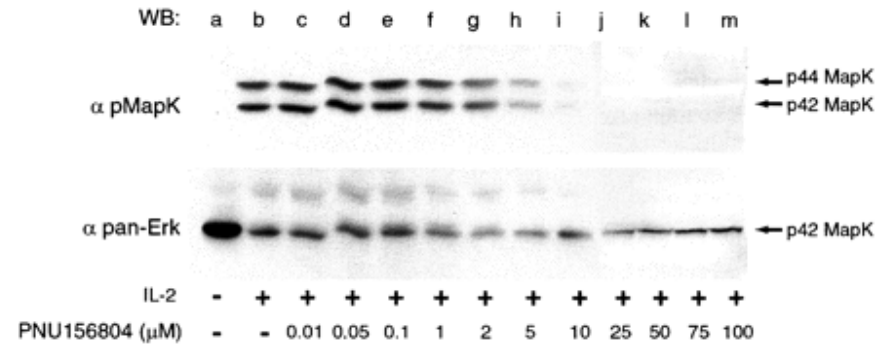


Figure 9. PNU156804 disrupts IL2-mediated p44/42 Erk1/2 phosphorylation. Quiescent PHA-activated T cells were treated with DMSO (control; lanes a-b) or increasing concentrations of PNU156804 for 16 hours and were stimulated in the presence of 100 nM IL2 at 37°C for 10 minutes. Cells were lysed, and total cell lysate was separated on 10% SDS-PAGE, transferred to PVDF membrane, Western blotted with antiphospho-p44/42 Erk1/2 (upper panel), stripped, and reprobed with pan-Erk antibody (lower panel). Arrows indicate the location of p44/42 Erk1/2.

CHAPTER 2

Uncoupling Stat5 activation from parallel signaling pathways in lymphoid cells

Introduction

As stated earlier, Stat5 is critical for immune regulation and T cell function. The binding of IL2 to its receptor results in rapid tyrosine phosphorylation of Stat5 by IL2R-associated Jak kinases [67]. Several hematopoietins share the IL2Rγ receptor subunit that serves as a common receptor for cytokines including IL2, IL4, IL7, IL9, IL13, IL15 and IL21. In addition to tyrosine phosphorylation, earlier work demonstrated that IL2 induces the rapid serine phosphorylation of both Stat5a and Stat5b in primary human and rat T cell lines [143, 144] and that this phosphoacceptor site is likely confined to the transactivation domain [85]. IL2 induces the activation of several serine/threonine kinases [20, 23, 28], which directly or indirectly may play a role in the serine phosphorylation of Stat5a and Stat5b and the mitogenic response.

In this chapter, we sought to identify the possible convergence of these signaling pathways on the level of serine phosphorylation of Stat5a/b, as well as their contribution to the IL2 mediated growth of lymphoid cells.

Results

IL2 differentially regulates serine phosphorylation of human Stat5a (S726) and Stat5b (S731). In order to detect serine-phosphorylated Stat5, site-specific phosphoserine antibodies to this domain were generated and specificity verified using phosphopeptide competition assays (data not shown). Next quiescent PHA-activated human T cells were stimulated with IL2 from 0 to 120 min; Stat5a or Stat5b was immunoprecipitated, separated on SDS-PAGE and ultimately blotted with phosphospecific Stat5a/b antibodies. Both Stat5a and Stat5b displayed nearly identical tyrosine phosphorylation kinetics, peaking at approximately 10 min when blotted with antiphosphotyrosine antibody (Figure 10A; upper panel). IL2 promoted Stat5a/b serine phosphorylation within their PSP-motif that was detected as doublets for each transcription factor (Figure 10A; middle panel). However, Stat5a serine phosphorylation was slightly protracted with maximal levels reached between 10-30 min, while serine phosphorylation peaked after approximately 30 min for Stat5b. At 60 min post IL2 stimulation, the slower migrating form of Stat5a showed pronounced

dephosphorylation and was weakly detectable at 120 min. In contrast both phosphorylated forms of Stat5b were detectable with little reduction in signal at 120 min. Stat5a/b immunopurified from non-IL2 treated T cells did not display any levels of tyrosine phosphorylation (Figure 10A, lanes a, g; upper panel), and only Stat5b showed a very weak level of serine phosphorylation (Figure 10, lane g; middle panel). Reprobing of both blots with a pan-Stat5 antibody confirmed equivalent loading (lower panel).

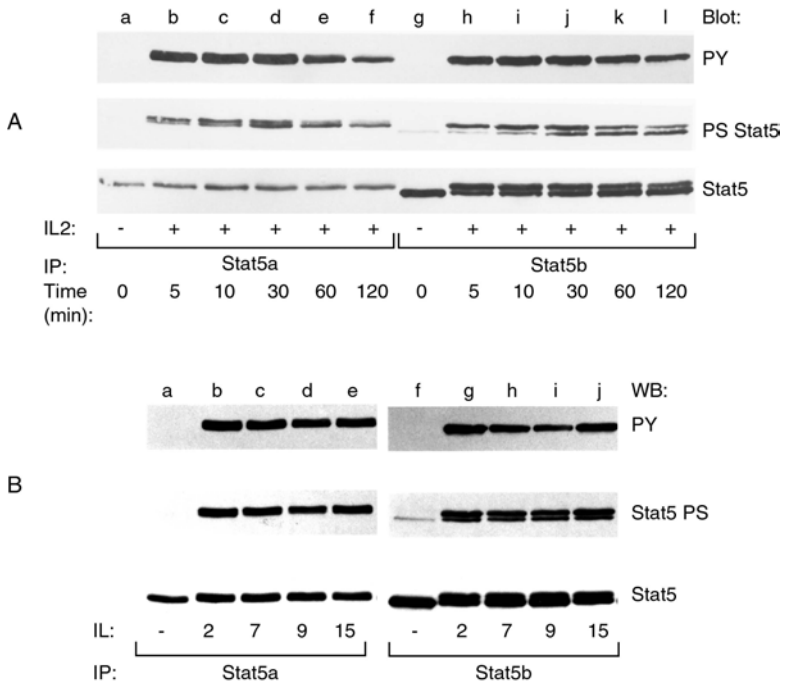


FIGURE 10. Cytokines utilizing γc induce Stat5a/b (726/731) serine phosphorylation. (A) Quiescent human T cells were incubated with medium (-) or 100 nM IL2 (+) at 37 °C for various times as indicated, and lysates were immunoprecipitated (IP) for Stat5a or Stat5b and blotted with α-phosphotyrosine (PY), α-phosphoserine Stat5 (PS Stat5) or α-Stat5 (Stat5) (B). Quiescent human T cells were incubated with medium (-) or 100 nM IL2, IL7, IL9 and IL15 (+) for 10 min at 37 °C. Cells were lysed and immunoprecipitated for Stat5a (lanes a, b, c, d and e) or Stat5b (lanes f, g, h, i and j). Samples were blotted with α-phosphotyrosine (PY), α-phosphoserine Stat5 (PS Stat5) or α-Stat5 (Stat5).

Multiple γc cytokines induce serine phosphoylation of Stat5a (S726) and Stat5b (S731) in T cells. To determine if the Stat5 proline directed serine kinase was uniquely activated by IL2 or also a shared serine kinase by this family of TCGFs, quiescent human T cells were stimulated with 100 nM of IL2, IL7, IL9 or IL15 for 10 min at 37 °C. Either Stat5a or Stat5b proteins were immunoprecipitated from corresponding cell lysates,

separated by SDS-PAGE and probed with α-phosphotyrosine (Figure 10B, upper panel), α-phosphoserine Stat5a/b (middle panel) or α-Stat5 (lower panel). Each growth factor showed comparable ability to promote Stat5a/b tyrosine and serine phosphorylation of each conserved residue.

Current crystallographic results of Stat1, 3 and 4 dimers are based on carboxyl-truncated forms that lack the transactivation domain that harbor the Pro-Met-Ser-Pro-motif and not known if the putative phosphoserine protrudes away from each monomer to the milieu to interact with transcriptional enhancing machinery, complementary Stat dimer or DNA [74, 146]. To test the notion that the native PSP site in Stat5a/b might be exposed and accessible to transcriptional cofactors, anti-phosphoserineStat5 antibodies were used in an EMSA to supershift the complex. As shown in Figure 11, nuclear cell extracts (5μg/well) were mixed with a [^{32}P]-labeled β-casein probe that is readily bound by activated Stat5a/b (lanes b and i, indicated by an arrow) could be verified by supershifting with antibodies to Stat5a (lanes c and j), Stat5b (lanes d and k), or both (lanes e and l). However, the phosphoserine Stat5a/b was also able to partially supershift the Stat5 complex (lanes f and m). The specificity was confirmed when normal rabbit sera failed to supershift the Stat5/DNA complexes (lane g and n). These findings suggest that either the phosphoserine site is exposed; albeit weakly on Stat5a/b or that a limited pool of unoccupied Stat5a/b phosphoserine (S726 or S731) is accessible to the phosphoserine Stat5a/b antibody.

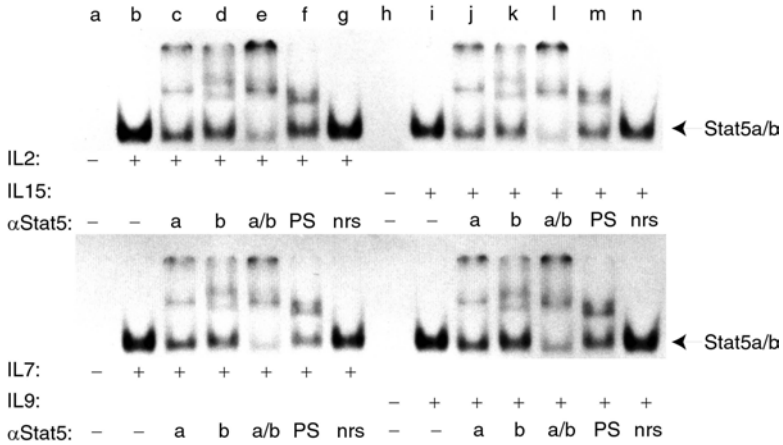


FIGURE 11. IL2 family cytokine-mediated Stat5 DNA binding is partially supershifted with phosphoserine-Stat5a/b antibodies. Quiescent human T cells were incubated with medium (-) or 100 nM IL2, IL15 (upper panel), IL7 and IL9 (lower panel) (+) for 10 min at 37 °C. Nuclear extracts corresponding to 5 μg of protein were incubated either with normal rabbit serum (lanes g and n), α-Stat5a (lanes c and j), α-Stat5b (lanes d and k), α-Stat5a plus α-Stat5b (lanes e and l), α-phosphoserine-Stat5 (lanes f and m) in combination with a [^{32}P]-labeled oligonucleotide probe corresponding to the PRL response element of the β-casein gene promoter. Arrow indicates migrational location of nonsupershifted Stat-DNA complex.

Phorbol-Esters but not αCD3 treatment of human T cells induces serine phosphorylation of Stat5a and Stat5b in Human T cells. Multiple TCGF receptors have been demonstrated to activate Stat5a/b tyrosine phosphorylation [143, 147, 148] while recent work showed that αCD3 stimulation of T cells rapidly activates Stat5a/b tyrosine phosphorylation and is critical for antigen induced T cell proliferation [149]. To test whether engagement of the TCR complex with αCD3 antibodies can activate a Stat5a/b proline directed serine kinase, PHA-activated T cells were made quiescent and then activated for 10 min in the presence of 5 μg/ml of αCD3 (Figure 12A, lanes b, d, j) or with 100 nM IL2 (lanes f, h). Three independent experiments failed to detect αCD3-mediated serine phosphorylation of Stat5a/b (middle panel) as well as phosphotyrosine Stat5 (Y701) using a Stat5 Y701 specific monoclonal antibody (upper panel). Activation of the TCR complex was verified by αCD3-induction of p56Lck tyrosine phosphorylation (lanes i, j), whereas IL2 mediated phosphorylation of both the conserved Stat5a/b tyrosine (Y699/701; upper panel) and serine (S726/731: middle panel) residues was detected. Reprobing of both blots with a pan-Stat5 or p56Lck verified equivalent protein loading (lower panel).

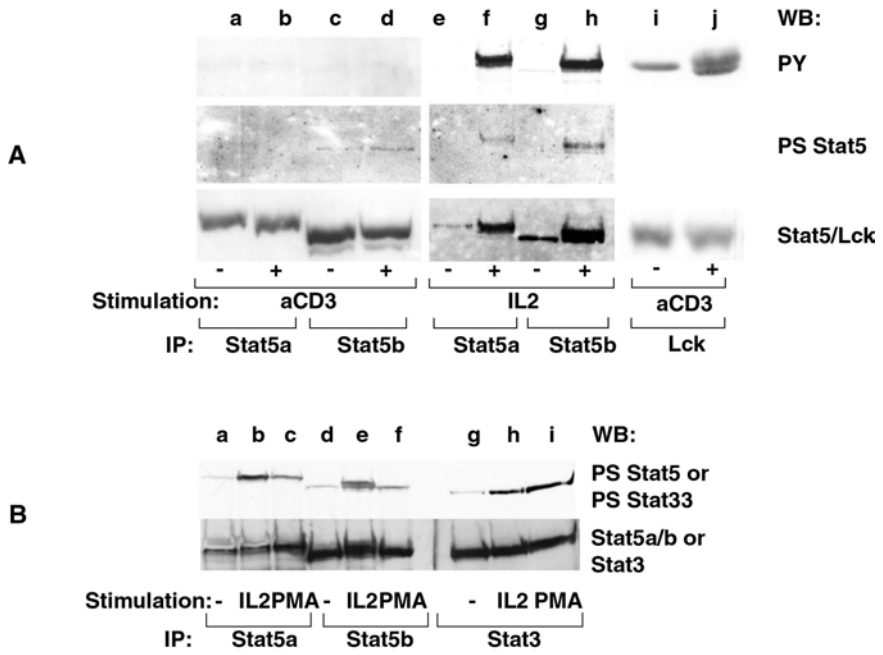


FIGURE 12. Phorbol-ester, but not αCD3, stimulation induces Stat5a/b (S726/731) serine phosphorylation in human T cells. (A) Quiescent human T cells were incubated with medium (-), 100 nM IL2 or 5 μg/ ml αCD3 antibody (+) for 10 min at 37 C, and lysates were immunoprecipitated (IP) with α-Stat5a (lanes a, b and e, f), α-Stat5b (lanes c, d and g, h). Samples were blotted for α-phosphotyrosine Stat5 (PY Stat5, upper panel), α-phosphoserine-Stat5 (PS Stat5, middle panel) and α-Stat5 (Stat5, lower panel). To confirm TCR engagement by αCD3 antibody, p56Lck was also immunoprecipitated from untreated (lane i) or αCD3 stimulated (lane j) and ultimately blotted with αPY (upper panel) or αLck antibody (lower panel). (B) Quiescent human T cells were incubated with medium (-), 100 nM IL2 or 1μg/ml PMA for 10 min at 37 °C, and lysates were immunoprecipitated (IP) with α-Stat5a (lanes a, b and c), α-Stat5b (lanes d, e and f) or α-Stat3 (lanes g, h and i). Samples were blotted for α-phosphoserine-Stat5 (PS Stat5) or α-phosphoserine-Stat3 (PS Stat3) shown in upper panel or α-Stat5 (Stat5a/b) and α-Stat3 (Stat3) for the lower panel. Migrational position is shown on the left.

Phorbol esters such as PMA have been shown to activate Stat3 serine phosphorylation be dependent on PKC-Raf-Mek cascade [72, 80]. To determine whether this pathway regulates the Stat5 serine kinase, quiescent human T cells were stimulated for 10 min with either 100 nM IL2 (Figure 12B; lanes b, e, and h) or 1μg/ml PMA (lanes c, f, and i). Cells were then lysed and immunoprecipitated with antibodies to Stat5a (lanes a-c), Stat5b (lanes d-f) or Stat3 (lanes g-i). Samples were blotted with either phosphoStat5a/b (S726/731) or

phosphoStat3 (S727) antibodies. While PMA did induce serine phosphorylation of the proline-flanked serine in Stat5a (lane c), Stat5b (lane f) and Stat3 (lane i), IL2 was generally more efficient. Equivalent Stat3 and Stat5a/b protein was verified by reprobing of all blots.

IL2-mediated Stat3, but not Stat5a/b serine phosphorylation is sensitive to inhibition of several IL2 activated Stat serine kinases. IL2 is a prominent activator of several serine-threonine kinases including Erk1/2, PI3-K and mTor, all have been established as Stat1/3 activators [75, 76, 81]. Herein we examined their role in promoting Stat3 serine phosphorylation in the IL2 responsive YT cell line (shown) (Figure 13). First, YT cells were stimulated in the presence of 100nM IL2 (Figure 3, lanes b-e) or 1μg/ml PMA (lanes g, h) and immunoprecipitated Stat3 blotted for phospho-tyrosine (upper panel) or -serine (middle panel) were found increased by either agent. Inhibition of Stat3 serine phosphorylation was detected following IL2 stimulated cells pretreated with 50 μM PD98059 (lane c), 20 nM rapamycin (lane d) or 100 nM wortmannin (lane e) compared to controls (lane b). Similarly, PMA-induced Stat3 S727 phosphorylation was inhibited following inhibition of Mek1 with PD98059 (middle panel, lane h).

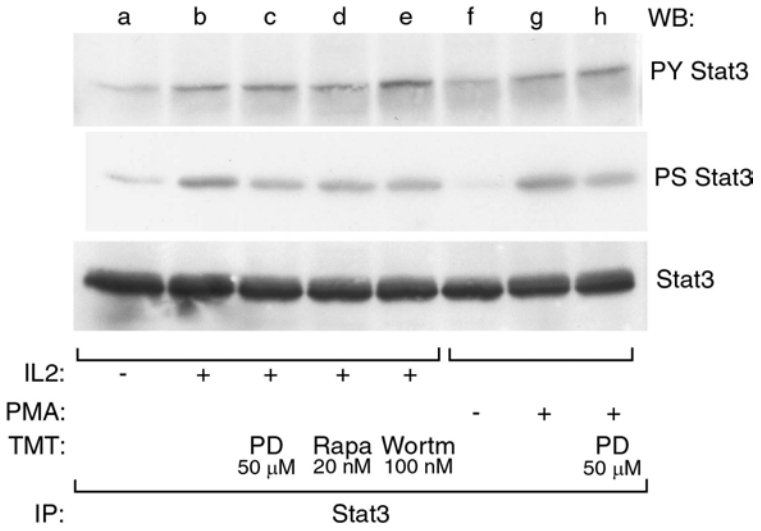


FIGURE 13. IL2-mediated Stat3 serine phosphorylation is inhibited by PD98059, wortmannin or rapamycin. YT cells were pre-treated for 1 hour at 37 ^{o} C without (lanes a, b, f, g) or with 50 μM PD98059 (PD; lane c, h), 20 nM rapamycin (Rapa; lane d), or 100 nM wortmannin (Wortm; lane e) that had been stimulated in the absence (lane a, g) or presence of 100 nM IL2 (lane b-e) or 1 ug/ml PMA (lanes g, h) for 10

min. Stat3 was immunopurified and blotted with antiphosphotyrosine (upper insert), Stat3 phosphoserine S727 (middle insert) or Stat3 (lower insert) antibodies.

To investigate the role of these kinases and/or their downstream counterparts in IL2-induced Stat5 serine phosphorylation, YT cells were treated with increasing amount of Mek1 inhibitor PD98059 (0-75 μM; upper panel), PI3K inhibitor wortmannin (0-500 nM; middle panel) or mTor inhibitor rapamycin (0-25 nM; lower panel) as shown in Figure 14. Cells were then incubated for an additional 10 min in the absence (-) or presence (+) of 100 nM IL2. Stat5a (lanes a-g) or Stat5b (lanes h-n) proteins were independently immunoprecipitated from the cell lysates and blotted with α-phosphotyrosine Stat5 (upper panels), α-phosphoserine Stat5 (middle panels) and α -Stat5 (lower panels). Compared with untreated control samples (lanes a, b, or h and i), pretreatment with each selective inhibitor displayed no significant deleterious effects on IL2-mediated Stat5a/b tyrosine or serine phosphorylation. It is concluded from these experiments that IL2 stimulated Stat5a/b serine kinase(s) acts independent from each of these serine kinase-signaling pathways.

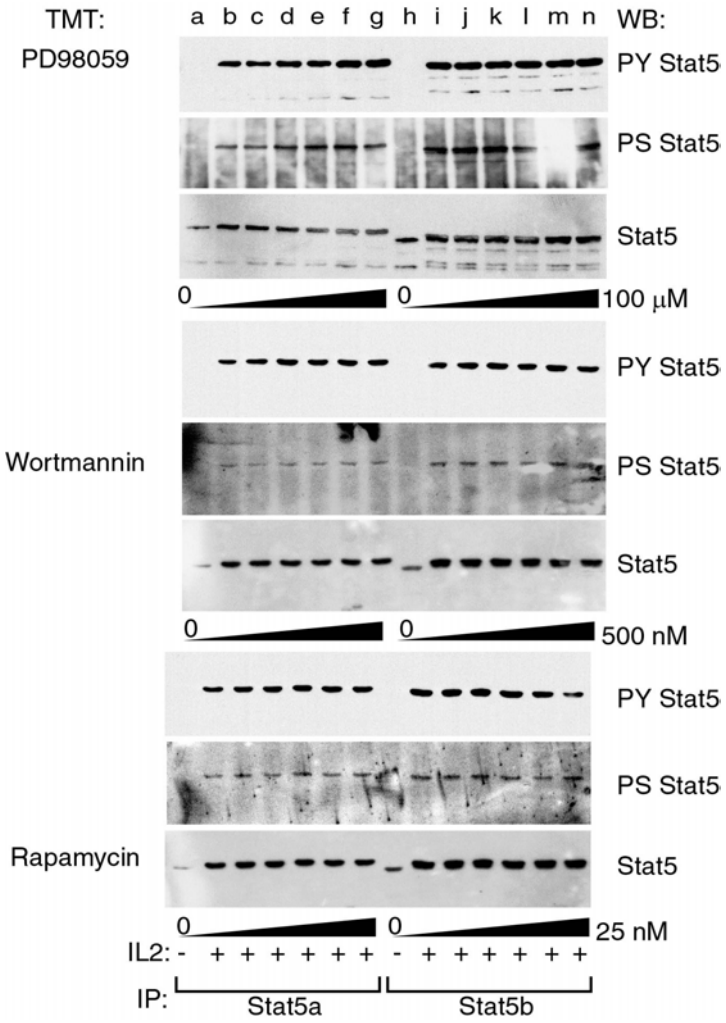


FIGURE 14. IL2 induced Stat5a and Stat5b phosphorylation on Ser726 and Ser731 residues are not affected by PD98059, wortmannin or rapamycin. YT cells were pre-treated for 1 hour at 37 ^{o} C without (lanes a, b, h, i) or with increasing amount of PD98059 (PD; lane c-g, j-n, upper panel), rapamycin (Rapa; lane lane c-g, j-n, lower panel), or wortmannin (Wortm; lane c-g, j-n, middle panel) then stimulated in the absence (lane a, h) or presence of 100 nM IL2 (lanes b-g, i-n) for another 10 min. Cells were lysed and immunoprecipitated (IP) with α-Stat5a (lanes a-g) or α-Stat5b (lanes h-n) antibodies. Samples were blotted for α-phosphotyrosine Stat5 (PY Stat5), α-phosphoserine Stat5 (PS Stat5) or α-Stat5 (Stat5).

IL2 promotes the autokinase activity of A-, B- and C-Raf isoforms. PMA can directly stimulate PKC, which in turn activates c-Raf and subsequently Erk1/2 [150]. One possible hypothesis to test was whether the Stat5a/b serine kinase bifurcates from Raf to an alternate proline directed Stat5a/b serine kinase. Although the Raf-protein kinases consist of three isoforms (A-, B-, and C-Raf) to regulate cell proliferation, differentiation, and

apoptosis, IL2 signaling studies have primarily focused on C-Raf [151, 152]. To address this question, human YT cells were stimulated with IL2 for 10 min and each Raf isoform immunoprecipitated from equivalent protein (1 mg) using a Raf specific or isotype control antibody and autokinase assay performed using γ- [^{32}P]-ATP (see Experimental Procedures for details). Depicted in Figure 15 is a representative autoradiograph (n=3) that showed A-Raf (68 kDa) and C-Raf (74 kDa) had no basal activity but was inducible upon IL2 stimulation (lanes b and h). B-Raf exhibited a high level of basal enzyme activity (lane d), as previously reported [153] and migrates at two positions within the gel (74 and 95 kDa) but can be further autophosphorylated following IL2 treatment (lane e). Reprobing of these membranes suggested each Raf isoform is equally expressed within YT cells (lower panel).

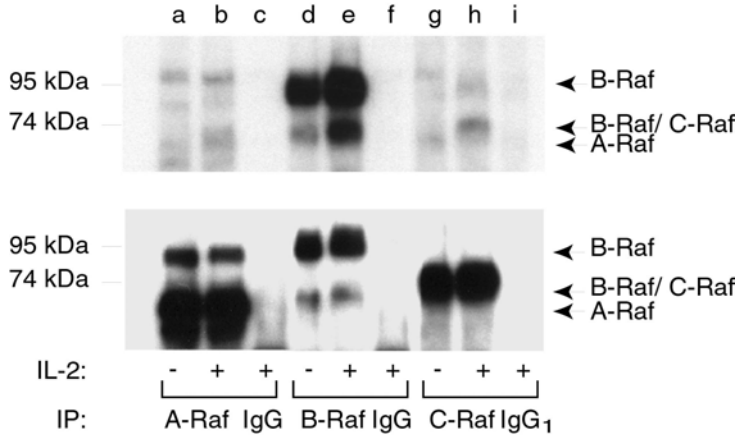


FIGURE 15. IL2 induces activation of three different isoforms of Raf in YT cells. Autophosphorylation of immunoprecipitated A-Raf (lanes a, b), B-Raf (lanes d,e) and C-Raf (lanes g,h) from YT cells incubated with (+) and without (-) IL2 (100 nM) for 10 min at 37 °C and subjected to an "in vitro" kinase assay. Western blot analysis for A-, B- and C-Raf is shown on lower panel. Migrational positions are shown on the left. Arrows on the right indicate different isoforms of Raf kinases. Normal rabbit serum (lanes c, f) and mouse IgG_1 (lanes i) were used as controls for immunoprecipitation.

Previous publications present evidence that C-Raf-1 becomes activated upon αCD3 antibody and IL2 treatment [151, 152], but the expression profile and the role of A- and B-Raf in lymphoid cells has not been extensively studied. First, we tested the expression pattern and the activation of Raf isoforms by IL2 and αCD3 antibody treatment in normal human T cells (Figure 16). Cells were made quiescent and stimulated with medium (-),

with αCD3 antibody (+) in panel A for 5 min or with IL2 (+) in panel B for 10 min at 37 ºC, then lysed and immune-complexes formed with A-, B- or C-Raf antibodies were subjected to an "in vitro" kinase assay using kinase-dead MEK1-GST as a substrate (Figure 16). Reprobing the blots with the corresponding Raf antibodies (Figure 16, panel A, lower insert) suggests that all Raf isoforms are equally expressed in normal human T cells. In order to test whether Raf activation induced by IL2 and αCD3 antibody correlates with Erk1/2 activation, 30 μg of the lysates from nonstimulated (-), IL2 and αCD3 stimulated (+) human T cells (described above) was separated on a 10% SDS-PAGE and blotted with phosphospecific antibody raised against the doubly phosphorylated/ active form of Erk1/2. IL2 and αCD3 treatment both promoted Erk1/2 phosphorylation in human T cells (Figure 16, panel C).

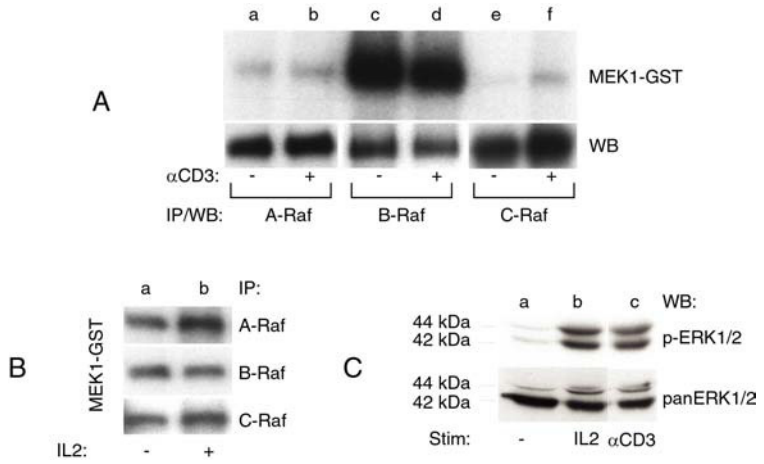


FIGURE 16. IL2 or αCD3 antibody treatment differentially activates Raf isoforms in human T cells. (A) Quiescent human T cells were incubated with medium (-) or 10μg/ml αCD3 antibody (+) for 5 min at 37 °C, and lysates were immunoprecipitated (IP) for A-, B- and C-Raf and subjected to an "in vitro" kinase assay. Membranes were blotted with anti-A-, B- and C-Raf antibodies to prove equal protein loading. (B) Quiescent human T cells were incubated with medium (-) or 100 nM IL2 (+) for 10 min at 37 ° C. Lysates were immunoprecipitated (IP) for A-, B- and C-Raf and subjected to an "in vitro" kinase assay. (C) Quiescent human T cells were incubated with medium (-), 100 nM IL2 for 10 min and 5μg/ml αCD3 antibody for 5 min at 37 °C. 30 μg of whole cell lysates were separated by SDS-PAGE and blotted with antibodies to phosphorylated ERK1/2. Reblot with anti-panERK is shown in the lower panel.

Antisense oligonucleotide treatment of A-, B-, or C-Raf inhibits protein expression and kinase activity. To determine a possible role for Raf isoforms in activating the

Stat5a/b serine kinase we sought to identify a specific inhibitor of each Raf product. Due to the absence of selective pharmaceutical inhibitors of each Raf enzyme, antisense oligonucleotides (ODN) were employed of 2'-methoxy-ethyl modified phosphorothioates previously shown to specifically inhibit each Raf isoform [102]. Dose curves verified that under optimized conditions, 5 μM of each antisense delivered via electroporation were sufficient to disrupt total Raf expression by no less than 50% (see Experimental Procedures). For this assay, YT cells were either untreated (Figure 17A, lanes a, b), electroporated without (lane c) or with 5 μM antisense to each Raf product (A-Raf upper, B-Raf middle and C-Raf lower panel; lane d) or nonsense control oligonucleotide (lane e). Within 48 hrs post-transfection cells were incubated with (+) or without (-) 100 nM IL2 for 10 min at 37 °C and subjected to an "in vitro" kinase assay using kinase-inactive MEK1-GST as a Raf substrate. Percent inhibitory index (% I. I.) indicates normalized data for kinase activity of each antisense versus nonsense control treated samples. Both protein level and kinase activity of A-, B- and C-Raf was decreased upon specific oligonucleotide treatments, typically greater than 70% (n=3).

To verify that the decrease in both enzyme activity and protein level was not the direct result of toxicity triggered by the antisense treatment, cell viability measured by Trypan Blue dye exclusion and determined to be greater than 80% (Figure 17A; n=3). To investigate whether each Raf antisense disrupts p44/42 Erk1/2 activity, cell lysates obtained from Panel A (Figure 17) were next examined for a loss in Mek1/2 phosphorylation of the TEY motif in Erk1/2 using anti-active antibodies (Panel B). 20 μg of total cell extracts from each treated sample were subjected to separation by SDS-PAGE (10% acrylamide gels), transferred to PVDF-membrane and blotted with phosphoErk1/2 antibodies. In contrast to nonsense oligonucleotide treatment (lane f), each antisense oligonucleotide reduced Erk phosphorylation by greater than 70% when normalized against p42 identified using a pan-Erk antibody. Western blots and densitometric analysis of normalization of Erk1/2 phosphorylation yielded an inhibitory index (I.I.) of 70%. Based on these results we conclude that activation of each of three Raf isoforms contributes to promote Erk1/2 activation.

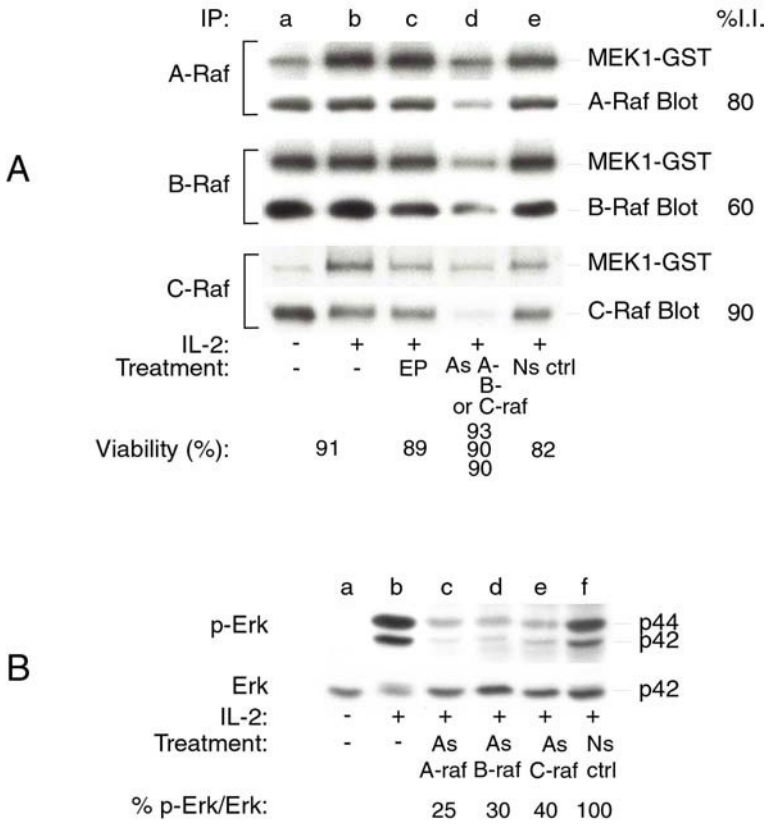


FIGURE 17. IL2 induced activation of different Raf isoforms are inhibited by antisense oligonucleotides. (A) YT cells were electroporated in the presence of 5 μM antisense oligonucleotide or nonsense control, left untreated or electroporated with no oligonucleotides. Cells were harvested and stimulated with (lanes b, c, d, e) or without (lane a) 100 nM IL2 for 10 min at 37 °C at 48 hours post-transfection. A-, B- and C-Raf immune complexes from equal amount of total cell lysates were prepared and subjected to an in vitro kinase assay using the purified inactivated MEK1-GST as a substrate. The resulting phosphorylated products were resolved on an 8% SDS-PAGE, transferred onto PVDF membrane and exposed to x-ray film at -70 °C. Western blots for A-, B- and C-Raf are shown on lower rows of each panel. Inhibitory Index: (I. I.) represents decrease in protein level as percentage of nonsense control treated sample. Lanes a, b: non-transfected, lane c: electroporated control, lane d: 5 μM antisense A-raf (upper panel), antisense B-raf (middle panel), antisense C-raf (lower panel), respectively, lane e: 5 μM nonsense control. (B) 48 h post-transfected YT cells were incubated with (+) and without (-) IL2 (100 nM) for 10 min at 37 °C. 30 μg of whole cell lysates were separated by SDS-PAGE and blotted with antibodies to phosphorylated Erk1/2. Reblot with anti-panErk is shown in the lower panel. Ratio of phosphorylated / unphosphorylated Erk protein is indicated (as % of nonsense control).

Inhibition of A-, B- and C-Raf with antisense oligonucleotides does not affect Stat5a and Stat5b serine phosphorylation. Since PMA or IL2 activates Raf kinases, we next

examined whether inhibition of each Raf isoform would block Stat5a/b serine phosphorylation (Figure 18). For this study, YT cells were electroporated with 5 μM A-, B- or C-Raf antisense oligonucleotides (lanes i and j, k and l, m and n, respectively), a mixture of 9 μM of Raf antisense oligonucleotides (lanes g and h), 9 μM nonsense control (lanes e and f), no oligonucleotides (lanes c and d) or left untreated (lanes a and b). Stat5a (upper panel) and Stat5b (lower panel) immune complexes were isolated from equivalent protein cell lysates and blotted with α-phosphotyrosine (upper inserts), α-phosphoserine Stat5 (middle inserts) and α-panStat5 (lower inserts) antibodies. Neither Stat5a nor Stat5b showed a decrease in IL2-induced serine/tyrosine phosphorylation upon the Raf antisense oligonucleotides treatment, suggesting that the IL2-activated Stat5 serine kinase does not require all three Raf isoforms, and possibly their downstream effectors Mek1/2, or Erk1/2 proteins/pathways.

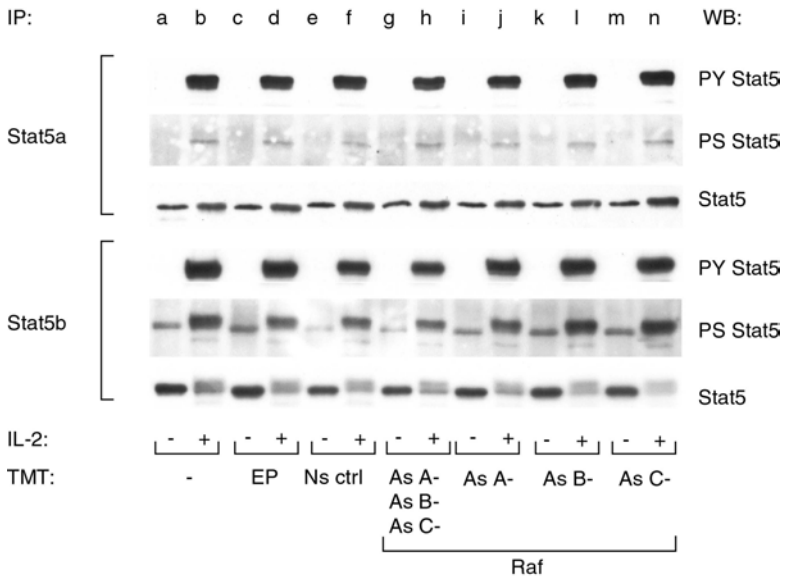


FIGURE 18. IL2 induced Stat5a and Stat5b phosphorylation on Ser726 and Ser731 residues is not affected by A-, B- and C-Raf antisense oligonucleotide treatment. 48 h post-transfected YT cells were incubated with (+) and without (-) IL2 (100 nM) for 10 min at 37 °C. Stat5a (upper panel) and Stat5b (lower panel) immune complexes from equal amount of total cell lysates were prepared. Samples were resolved on a 7.5% SDS-PAGE, transferred onto PVDF membrane and blotted with α-phosphotyrosine-Stat5 (PY Stat5), α-phosphoserine-Stat5 (PS Stat5) or α-Stat5 (Stat5) as indicated. Non-treated control (lanes a, b), electroporated control (lanes c, d), 9 μM nonsense control (lanes e, g), 5 μM A-Raf (lanes i, j), 5 μM B-Raf (lanes k, l), 5 μM C-Raf antisense (lanes m, n) ODN, respectively. Triple treatment with 3 μM of each of A-, B- and C-Raf antisense (lanes g, h) oligonucleotides.

Effect on IL2-induced cell proliferation following inactivation of Raf isoforms, Mek1/2, mTor or PI3K. Lymphocytes harvested from Stat5a/b gene deficient mice fail to proliferate in response to IL2 stimulation [154]. These findings suggest that Stat5a/b represent critical regulatory molecules required to mediate TCGF-dependent cell cycle progression. To examine the contribution of the IL2-induced effector pathways to IL2-mediated cell growth two cell lines were employed, the IL2 dependent murine cell clone CTLL-2 and human NK cell line, YT. Next both cell lines were treated with 5 μM A-, B-, and C-Raf antisense or nonsense control (Figure 19, panel A and B). 24 hrs post-transfection cells were cultured for another 16 hrs in the absence or presence of 1nM IL2 and then pulsed for the remaining 4 hrs with [^{3}H]-thymidine. [^{3}H]-thymidine incorporation during cell proliferation was measured from both treated cell lines. IL2 induced/basal level of thymidine incorporation was normalized against nonsense control oligo treated sample. From this typical experiment (n=3) YT cell proliferation driven by IL2 was generally reduced by no greater than 20% upon treatment with antisense to A- or B-Raf oligo, with C-Raf antisense treated cells more effectively inhibited, only approaching 30%, yet statistically significant ($p<0.05$) (Panel A). Raf antisense treatment of CTLL-2 (Panel B), displayed a similar response pattern to IL2 while within antisense, only C-Raf showed statistically significant inhibition ($p<0.05$) of cell growth.

These above-mentioned results suggest that inhibition of Raf isoforms had little effect on Stat5a/b activation and cell growth. Since inactivation of more classical cell proliferative pathways Mek, PI3K and mTor, had no affect on Stat5a/b phosphorylation, we next examined their action on the cell growth of these same cells. Thus, YT (Figure 19C) or CTLL2 cells (Figure 19D) were admixed with increasing concentrations of the Mek1/2 inhibitor PD98059 (10-75 μM) for 16 hrs in the absence (white bars) or presence (black bars) of 1 nM IL2. For both cell lines, PD98059 inhibited IL2-induced proliferation with less than 50% efficiency compared to vehicle treated (DMSO) sample. While the EC_{50} of this drug is ~10 μM, several fold higher concentrations were typically required to inhibit the IL2-mediated cell proliferation. Wortmannin (25-500 nM) or rapamycin (1-100 nM) showed even less inhibition on cell growth. In fact YT cells displayed a stimulatory effect while CTLL-2 cells were only weakly inhibited (less than 25%) when either

pharmaceutical was employed at concentrations nearly 5-fold their respective EC_{50} values. These results suggest that IL2 and other TCGFs activated Stat5a/b serine kinase pathway is functionally distinct and likely to act parallel to mTor, Mek and PI3K pathways that are dispensable for cell growth, at least within the time frame measured (< 24 hrs).

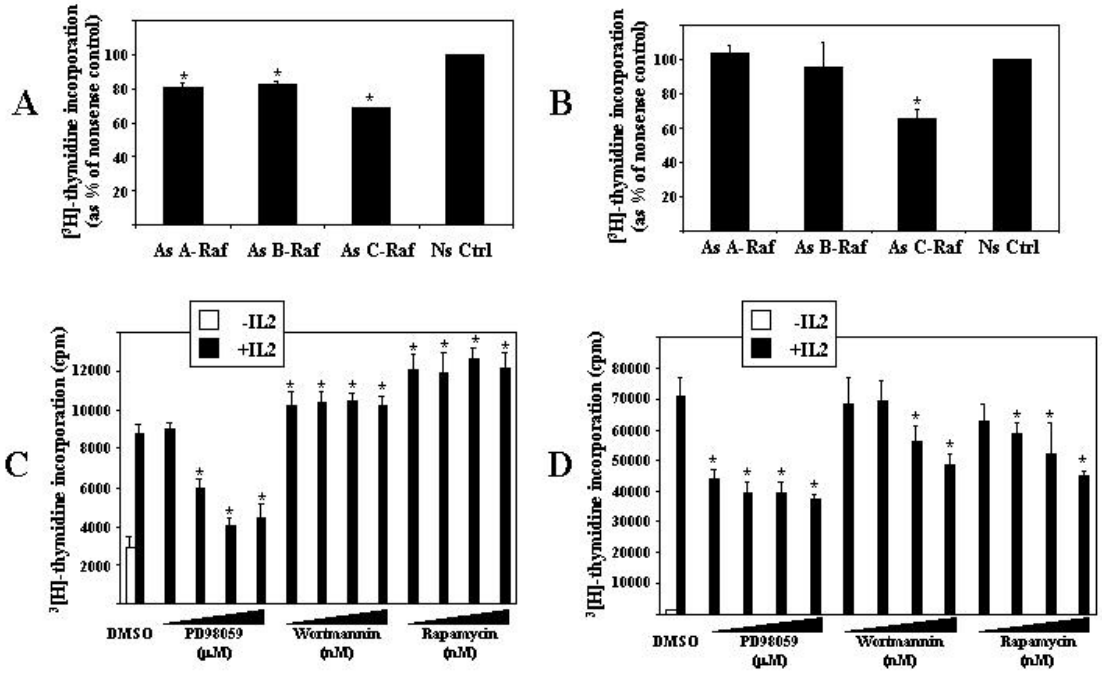


FIGURE 19. Effect of inhibitors of IL2 activated serine/threonine kinases on IL2 induced cell proliferation. After 24 h treatment with A-, B- and C-Raf antisense, quiescent YT cells (A) or CTLL2 cells (B) were plated in 96-well plates ($50x10^3$/well) and cultured in the presence (+) or the absence (-) of 1 nM IL2 for 16 h at 37 °C. Cells were then pulsed for the remaining 4 h with [^{3}H]-thymidine (0.5 μCi/200 μl; n=6). IL2 induced/basal level of incorporation of radiolabeled probe is plotted on the abscissa and expressed as % of nonsense control (average of three independent experiments). Proliferation of quiescent YT cells ($50x10^3$/well) (C) and CTLL2 cells (D) was examined after treatment with increasing concentrations of PD98059 (10, 25, 50, 75 μM), wortmannin (25, 100, 250, 500 nM) and rapamycin (1, 10, 25, 100nM) for 16 h at 37 °C in the presence or absence of 1 nM IL2. Cells were then pulsed with [^{3}H]-thymidine (0.5 μCi/200 μl; n=6) for 4 h and IL2 induced incorporation (cpm) of radiolabeled probe plotted on the abscissa.

CHAPTER 3

Selective disruption of constitutively active Stat3 in a human NK-like tumor cell line

Introduction

Current models suggest cytokines activate Stats to regulate T cell survival, one supported by our earlier findings. To further support this notion, we next addressed what would occur if constitutively active Stat molecules, in the absence of cytokines, were disrupted in lymphoid tumors.

In this chapter, we sought to investigate whether constitutive activation of Stat3 provides a cell survival signal in a lymphoid tumor cell line (YT). YT is a human NK-like cell line originally derived from a patient with Acute Lymphoblastic Lymphoma [155].
To block persistent Stat3 activation, we chose to use phosphorothioate antisense oligonucleotides with 2'-MOE wings at both the 3' and 5' ends, delivered by electroporation. As a control, a 5-nucleotide mismatched oligo with the same modification was utilized.

Results

Stat3 is constitutively active in YT cells. To demonstrate constitutive, but IL2-inducible, activation of Stat3 in this cell line, YT cells were stimulated with a 100nM IL2 (+) or left untreated (-) for 10 min at 37 ºC, lysed and Stat3 was captured with anti-Stat3 antibody. Western blots with anti-phosphotyrosine and anti-phosphoserine-Stat3 antibodies were performed. To verify equal protein loading, blot was reprobed with anti-Stat3 antibody. The basal phosphorylation level of both the conserved tyrosine and serine residues (Figure 20A, lane a, middle and upper insert, respectively) indicate that Stat3 is persistently activated in these cells. IL2 was able to promote further phosphorylation of these residues (Figure 20A, lane b, middle and upper inserts).

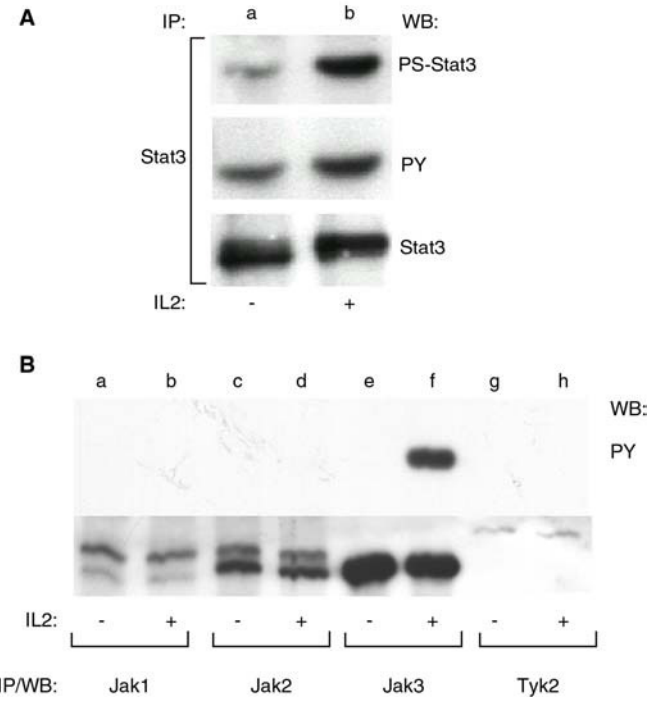


FIGURE 20. (A) **Stat3 is constitutively phosphorylated on Y701 and S727, which is further inducible by IL2.** YT cells were incubated with medium (-) or 100 nM IL2 (+) for 10 min at 37 ºC. Lysates were immunoprecipitated (IP) with anti-Stat3 antibody, subjected to SDS-PAGE and blotted with anti-phosphoserine- Stat3 (αPS; upper panel), anti-phosphotyrosine (αPY; middle panel) or αStat3 (lower panel) antibody. (B) **Jak tyrosine kinases are not constitutively activated in YT cells.** Cells were incubated with medium (-) or a 100 nM IL2 (+) for 10 min at 37 ºC. Lysates were IP with antibodies against Jak1, Jak2, Jak3 or Tyk2, subjected to SDS-PAGE and blotted with αPY antibody. Equal loading was confirmed by reprobing the blots with each Jak antibody.

Jak tyrosine kinases are not responsible for persistent Stat3 activation in YT cells. The regulation of Stat activity is very diverse, including upstream kinases and downstream negative effectors. Jaks are believed to be the primary upstream tyrosine kinases of Stats in many cytokine, hormone and growth factor signaling pathways. It has previously been recognized that disruption of constitutively active Jak activity by selective inhibitors (such as AG490) can block Stat activation [156]. In order to investigate whether these kinases are responsible for maintaining the basal phosphorylation/activation level of Stat3 (Figure 20B), YT cells were stimulated with medium (-) or IL2 (+) for 10 min at 37 ºC and lysed. Immune-complexes with anti Jak1 (lanes a, b), Jak2 (lanes c, d), Jak3 (lanes e, f) and Tyk2 (lanes g, h) antibodies were formed, followed by Western blot performed with anti-

phosphotyrosine antibody. Equal protein loading was verified by reprobing all blots with corresponding antibodies (lower panel). As shown on Figure 20B upper panel, none of the Jak kinases were active in the non-stimulated cells but Jak3 was inducible upon IL2 stimulation. We conclude that Jak tyrosine kinases do not serve as mediators of Stat3 hyperactivation in this cell line.

Antisense to Stat3 blocks constitutive Stat3 but not IL2-induced Stat5 DNA binding. Since several attempts failed to identify any other upstream activator of Stat3 in YT cells, we decided to directly target this molecule to determine its contribution to the transformed phenotype. We chose to utilize selective Stat3 antisense oligonucleotides to disrupt its activity. First, electrophoretic mobility shift assay was carried out to test whether Stat3 antisense treatment can efficiently block Stat3 DNA binding (Figure 21). YT cells were left untreated (lane a), electroporated with no oligonucleotide (lane b), 5 and 10μM of antisense oligo (lanes c, d) or 10μM mismatched control (lanes e-h), and cultured for 72 hrs. At this time point, cells were incubated with medium (-) or 100nM IL2 (+) for 10 min at 37 ºC. Nuclear extracts corresponding to 5 μg of protein were incubated with either normal rabbit serum (nrs; lane h) or α-Stat3 (lane g) in combination with a [^{32}P]-labeled DNA probe corresponding to the c-*fos* promoter *sis*-inducible (SIE) element m67 (panel A). Nuclear extracts from identical samples were incubated either with normal rabbit serum (lane h) or α-Stat5 (lane g) in combination with a [^{32}P]-labeled Stat5 oligonucleotide probe corresponding to the PRL response element of the β-casein gene promoter (panel B). As shown in Figure 21, antisense to Stat3 successfully disrupted constitutive Stat3 DNA binding compared to the mismatched control treated or only electroporated samples, but failed to affect IL2-induced Stat5 DNA binding. These results indicate that no significant nonspecific toxic effect occurred upon the oligonucleotide treatment.

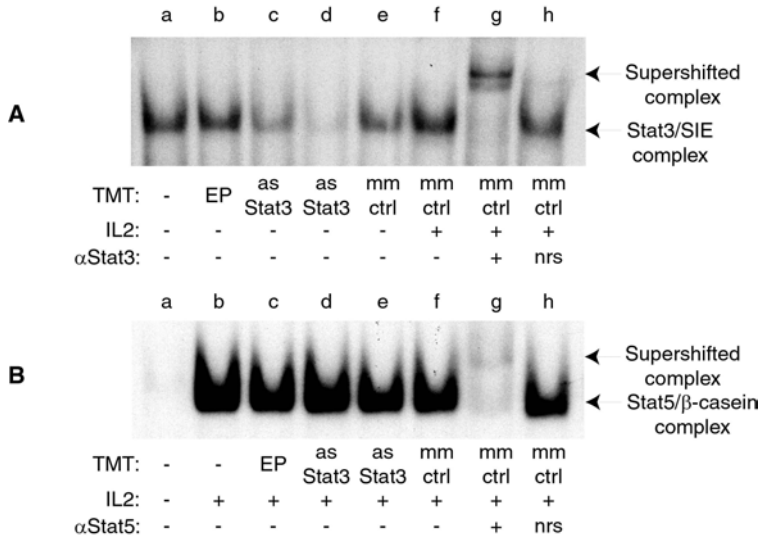


FIGURE 21. Selective antisense blocks Stat3 but not Stat5 DNA binding. YT cells were electroporated with 5 or 10 μM Stat3 antisense oligo (as Stat3), 10 μM mismatched control oligo (Mm Ctrl), with no oligo (EP) or left untreated (-) as indicated and then cultured for 72 hrs. Cells were incubated in the absence (-) or presence (+) of 100 nM IL2 for 10 min at 37 ºC. Nuclear extracts corresponding to 5 μg of protein were incubated with either normal rabbit serum (nrs; lane h) or α-Stat3 (lane g) in combination with a [^{32}P]-labeled DNA probe corresponding to the c-*fos* promoter *sis*-inducible (SIE) element m67 (upper panel) Nuclear extracts from identical samples were incubated either with normal rabbit serum (lane h) or α-Stat5 (lane g) in combination with a [^{32}P]-labeled Stat5 oligonucleotide probe corresponding to the PRL response element of the β-casein gene promoter (lower panel). Arrow indicates migrational location of nonsupershifted Stat-DNA complex.

Selective disruption of Stat3 induces apoptosis. Since Stat3 signaling controls transcription of genes that regulate cell growth and survival [59], we sought to test whether abrogated Stat3 activity interferes with these cellular events. As shown in Figure 20, YT cells were treated with 10 μM Stat3 antisense (upper right panel), mismatched control (lower left panel), or no oligonucleotide (upper left panel), and at 48 hrs post-transfection the cells were deposited on slides by standard cytospin procedure, fixed in 4% paraformaldehyde and incubated with the TdT endlabeling cocktail, and counterstained with propidium-iodide (Figure 22). Lower right panel shows quantification of the slides, by counting the TUNEL positive and propidium-iodide positive cells on at least three independent fields. The values plotted on the y-axis represent the average of the % of apoptotic cells per field. The number of apoptotic cells increased up to 35% in the

antisense Stat3 treated cells while remained under 10% in the electroporated or control oligo treated samples. From these results we conclude that the contribution of constitutively active Stat3 to the malignant phenotype of these cells is likely via suppressing apoptosis.

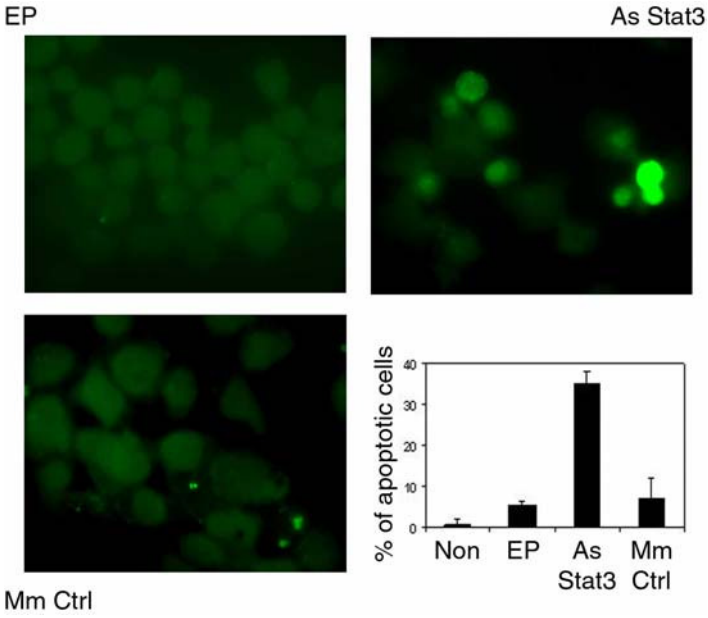


FIGURE 22. Selective disruption of Stat3 induces apoptosis. YT cells were electroporated with antisense Stat3 ODN as described above and cultured for 48 hrs. Cells were deposited on slides by standard cytospin procedure, fixed in 4% paraformaldehyde and incubated with the TdT end labeling cocktail, and counterstained with propidium-iodide. (Representative of two independent experiments, visualization by fluorescence microscopy.)

Antisense to Stat3 decreases viability that can be rescued by culturing the cells in IL2. IL2 activates several signaling cascades responsible for cell proliferation and survival (reviewed in [7]). YT cells are responsive to IL2, expressing the intermediate affinity IL2 receptor complex (IL2Rβ and γ). In order to test whether IL2 can induce signaling pathways that can compensate for the loss of Stat3, YT cells were transfected with antisense to Stat3 (described above), and at 24 hrs post-transfection transferred into medium containing 2% FCS in the absence (-) or presence (+) of 1nM IL2. A representative of three independent experiments is shown in Figure 23. As a result of the antisense treatment, viability was decreased with 50% and 70% at 72 (upper panel) and 96 hrs (lower panel) post-transfection, respectively, but culturing the cells in the presence of

physiological amount of IL2 reduced cell death to only 25%. This means that IL2 induced pathways can rescue cells from death triggered by suppressed Stat3 activation.

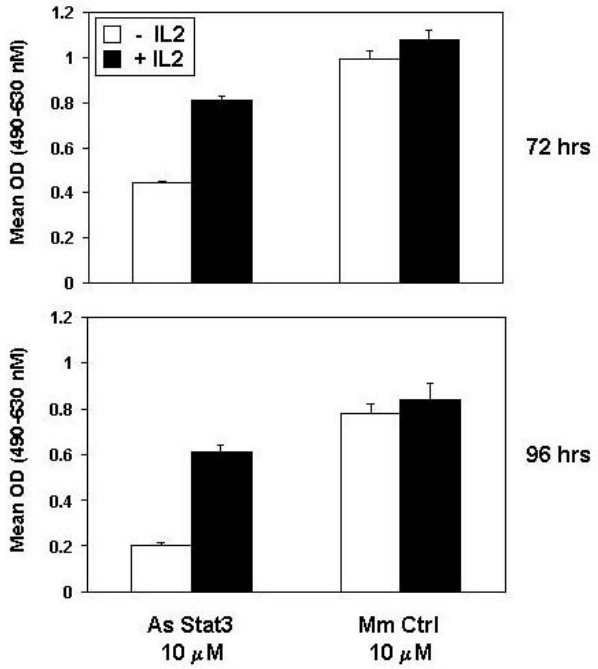


FIGURE 23. IL2 rescues Stat3 depleted YT cells. YT cells were electroporated in the presence of 10μM antisense oligonucleotide (As Stat3) or mismatch control (Mm Ctrl) as indicated. At 24 h post-transfection cells were transferred into growth medium containing 2% FCS in the absence (white bars) or presence (black bars) of 1 nM IL2 and cultured for another 48 h. 50 x 10^3 cells were seeded into 96-well plates and viability was determined at various time points by MTS assay as described in Experimental Procedures.

Culturing with IL2 induces tyrosine hyperphosphorylation of Stat5 in Stat3 deleted cells and upregulates Bcl-2. Upon IL2 binding, Stat5 becomes phosphorylated and migrates to the nucleus where activates transcription of genes known to be involved in the regulation of cell growth and survival [1, 13, 15]. We next wanted to test whether any correlation exists between the IL2 mediated survival of Stat3 deleted YT cells and the IL2 mediated activation of Stat5. Cells obtained from the previous experiment (Figure 23, upper panel) were lysed and immunoprecipitated for Stat3 (Figure 24A), Stat5a and Stat5b (shown), resolved by SDS-PAGE and Western blotted with indicated antibodies (Figure 24A). Antisense to Stat3 significantly reduced Stat3 protein expression, and as expected, tyrosine phosphorylation compared to the mismatched control treated or only electroporated samples. In the presence of IL2 (lanes e-h), Stat5 became tyrosine

phosphorylated. In the antisense treated cells (lanes f, g) a very pronounced hyperphosphorylation of both Stat5a and b (shown) was observed compared to the electroporated (lane f) or mismatched oligo treated samples (lane h) suggesting a compensatory role for Stat5 in the absence of Stat3 upon addition of IL2 into the culturing medium. One anti-apoptotic effector protein, Bcl-2, increases with IL2 treatment (Figure 24B) and possibly protects against apoptosis in Stat3 depleted YT cells.

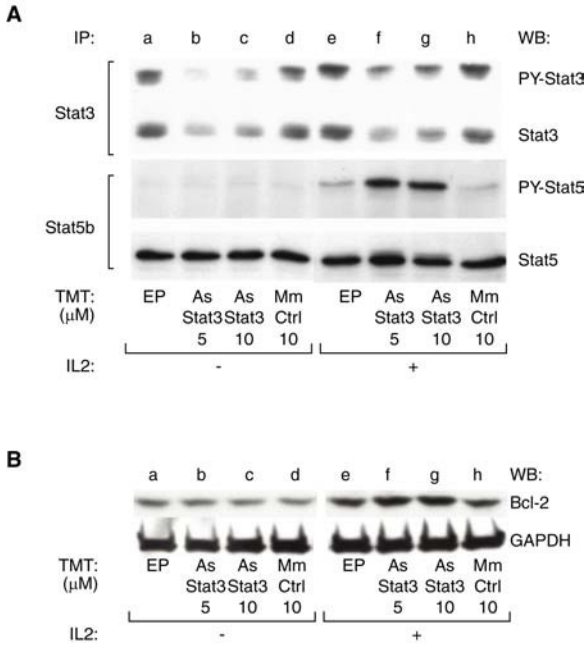


FIGURE 24. Culturing the cells with IL2 induces hyperphosphorylation of Stat5 in Stat3 deleted cells. (A) YT cells were treated as described above. At 24 h post-transfection cells were transferred into growth medium containing 2% FCS in the absence (-) or presence (+) of 1 nM IL2 and cultured for 48 hrs. Stat3 and Stat5b immune complexes from equal amount of total cell lysates were prepared, resolved on 7.5% SDS-PAGE and blotted with antibodies to PY-Stat3 or PY-Stat5. Corresponding reblots with Stat3 and Stat5 antibodies are shown below the PY blots. (B) **Stat5 hyperactivation correlates with Bcl-2 upregulation.** From 20μg of cell lysates obtained from Panel A Western blot was performed with anti-Bcl-2 and anti-GAPDH antibody was used to normalize the data.

VII. Discussion

The primary goal and objective of this study was to determine the role of possible parallel cell signaling pathways in maintaining lymphoid cell survival, primarily focusing on the Jak/Stat signaling pathway. Our rationale was that mice deficient in γc and its associated Jak3 tyrosine kinase and downstream substrates, Stat5a/b transcription factors, are immune suppressed due to non-functional T cells [1-4, 40]. We proposed that this condition is likely due to the redundant role of γc/Jak3 signaling and represents an essential first step in pathways activated by the family of γc cytokines that regulate lymphoid cell differentiation, growth and survival [16, 42]. To test our central hypothesis we investigated the contribution of these pathways to maintaining cell survival by selectively inhibiting them. The challenge of undertaking this project was the development of novel tools and reagents to inactivate these molecules. Our approach was to develop and then utilize pharmacological inhibitors or selective antisense oligonucleotides to determine the role of these specific molecules.

The first series of experiments studied the effects of inhibiting γc expression using phosphorothioate antisense oligonucleotides or Jak3 activation via a novel pharmacological inhibitor PNU156804.

In the second chapter, we tested whether IL2-mediated signaling pathways, all of which have been implicated in the growth/survival of lymphoid cells, actually converge at the level of Stat5 regulation. Lastly, we tested whether constitutively active Stat3, in the absence of cytokines, provides a cell survival signal in a lymphoid tumor cell line by directly targeting this protein with selective antisense oligonucleotides. It was hoped that at the end of the research we could determine the relative importance of the Jak/Stat pathway as opposed to the Jak/Mapk or Jak/PI3K in maintaining lymphoid cell survival.

Effects of selective inhibition of γc receptor and Jak3 on lymphoid cell survival

To initiate these studies, we first determined the effects of disrupting the expression of γc in a human NK-like tumor cell line YT, by using 17-mer phosphorothioate antisense

oligonucleotides; comparing all effects to the scrambled oligo treated or electroporated control samples.

Once we identified a selective gamma chain inhibitor, tests were initiated to determine the effect of deleting this protein on lymphoid cell processes. Our findings were:

(1) Antisense to γc (15μM) delivered via electroporation successfully inhibited protein expression (40%; Figure 5) at 72 hrs post-transfection.
(2) Bcl-2 protein level was unchanged.
(3) This treatment decreased the cell viability (40%) and increased the number of apoptotic cells (30%) compared to the scrambled control treated cells at kinetically equivalent points (Figures 6 and 7A).
(4) Disruption of the γc induced cell cycle arrest at 36 hrs following electroporation and increased the number of cells in subG$_1$ (15%) and G$_2$-M (12%) phases, and decreasing in G$_{0-1}$ (21%) (Figure 7B).

Results of Jak3 inhibition utilizing a novel pharmacological inhibitor PNU156804:

(1) PNU156804, but not the inactive control compound PNU159744, blocks the activation of Jak3 substrates (Stat5a and b), as assessed by phosphotyrosine and phosphoserine Western blots (Figure 8).
(2) PNU156804 disrupts the activity of p44/Erk1 and p42/Erk2 Ser/Thr kinases (Figure 9).

Long-term use of PNU156804 induces T cell apoptosis (~ 40%) at 48hrs (data not shown, personal communication with R. A. Kirken).

The γc receptor is central to cytokine mediated T cell proliferation [8]. A large body of literature has accumulated over the past few years that has attempted to reveal its exact role in cytokine signaling. However, most findings are derived from murine gene deletion experiments. In order to unveil γc function in *human mature* cells, the application of antisense oligonucleotides was invaluable to this process. As we presented here, antisense targeted against γc successfully inhibited protein expression. There are several advantages in the use of antisense oligonucleotides such as the sequence specificity and relatively low cost of the synthesis of the oligos. It provides a powerful technique for the

short-term modulation of the expression of the gene of interest, once an optimal target sequence is found and a successful method for delivery is optimized. However, as with all other techniques, antisense approaches have some inherent risks. The disruption of the target mRNA or the blockade of the target protein expression may not be complete. The occurrence of some non-antisense effects cannot be ruled out, although the application of appropriate controls with the same modifications can minimize some of these problems. Therefore, it is important to confirm observations made with antisense oligonucleotides by other independent techniques, such as monoclonal antibodies, dominant negative constructs, overexpression of the protein-of-interest, knockout mice or small molecule inhibitors. All of these techniques have their own limitations. For example, monoclonal antibodies bound to cell-surface molecules have the potential to initiate a signal through that molecule, while overexpression of a protein may cause low affinity interactions (which do not normally occur under physiological conditions between native proteins), or compensatory changes that might arise during the development of genetically altered mice.

The above results suggested that activation of hyperactive T cells found in allergy, asthma or allograft rejection could be corrected by the application of γc antisense, or at least targeted inhibition of this protein. Given the above-mentioned technical hardships of antisense therapy new methods of delivery are required to make it a relevant therapeutical treatment strategy. Nonetheless, the fundamental problem for all currently available immunosuppressants is the ubiquitous distribution of their targets, which ultimately results in toxicity [45, 46]; therefore we must continue to identify highly specific agents that can inactivate molecules uniquely expressed in resting T cells (e.g. Zap70) or in activated T and B cells (e.g. Jak3) and validate these potentially novel targets through in vitro and in vivo studies.

Several recent studies suggest that the Jak3 tyrosine kinase is an essential signaling intermediate for the development and function of T, B, and NK cells [47, 48]. Indeed, retroviral introduction of the Jak3 enzyme into Jak3-deficient mice restores normal T cell development [157]. Although understanding the signaling pathways activated by Jak3 (directly or indirectly) is far from complete, Jak3 signaling by Stat5a/b is necessary to regulate genes required for cellular proliferation [1]. As shown here, PNU156804 abolished IL2-dependent Stat5a/b tyrosine phosphorylation and activation. Interestingly,

blockade of Jak3 by PNU156804 also inhibited the phosphorylation of a conserved proline-serine-proline (Pro-Ser-Pro) motif in Stat5a and Stat5b and induced cell death within 48 h. Given the limited pattern of Jak3 expression, the γc-Jak3-Stat5 pathway is likely to represent a convergence point by which TCGFs drive T cell clonal expansion, thereby making it a preferred pathway for novel and selective immunosuppression. The limitation of validating this model is which Jak3 substrate is critical for regulating T cell activity. While it is known that Jak3 regulates Stat5, Mapk and PI3K signaling pathways, is one more crucial than the other for maintaining cell survival?

Uncoupling Stat5 activation from parallel signaling pathways in lymphoid cells

To address this question, we determined whether disruption of PI3K, Mapk and Raf pathways blocks Stat5 activity, a Stat5 serine kinase and possible effects on lymphoid cell growth.

The results of these studies are as follows:

(1) Serine phosphorylation of Stat5a-S726 and Stat5b-S731 occurs within a PSP-motif that is regulated by multiple TCGFs including IL2, IL7, IL9, or IL15 (Figure 10, 11).

(2) While Stat5a/b serine phosphorylation was not inducible following cross-linking of the T cell receptor via αCD3 ligation, it was readily activated by PMA in the absence of tyrosine phosphorylation (Figure 12).

(3) While several Stat dependent serine kinases including PI3K, Erk1/2 and mTor regulate Stat1, 3 and 4 phosphorylation, this positionally conserved serine residue in Stat5a/b does not appear to be one of their targets (Figure 13, 14).

(4) Novel evidence was presented that IL2 is competent to activate all three Raf-isoforms (A-, B-, and C-) (Figure 15, 16), but that their inactivation (Figure 17) failed to influence Stat5a/b (S725/S731 or Y699/701) phosphorylation (Figure 18).

(5) Based on [^{3}H]-thymidine uptake assays, inhibition of each of these serine kinases failed to significantly affect cytokine-mediated cell proliferation of human NK and murine T cell lines within the time frame tested (Figure 19).

Given the present findings that Stat5a/b activation remains intact upon disruption of Mapk, Raf and PI3K pathways, we conclude that these transcription factors are dependent on Jak3 but not on several IL2 mediated serine/threonine kinase pathways.

Current cytokine signaling models hold that ligation of cytokine receptors recruit and activate Jak enzymes that subsequently mediate phosphorylation of Stat proteins on a conserved tyrosine residue [158]. Herein we observed rapid tyrosine phosphorylation kinetics of Stat5a/b that closely mimic IL2-induced Jak3 autophosphorylation in human T lymphocytes (5-10 min) post TCGF stimulation (Figure 10A) [42, 159, 160]. The limited region of sequence divergence existing within Stat5a/b is localized to their transactivation domains that accommodate the conserved PSP-motif. While Stat5a/b share 95% sequence identity, they display differences in biological activity since mice void of Stat5a develop mammary gland defects [161] while Stat5b null mice manifest a runted phenotype [12]. The current work adds to a series of Stat5a versus Stat5b dissimilarities also localized within the transactivation domain, since both displayed different serine phosphorylation kinetics. IL2 treatment induced a serine phosphorylated Stat5a doublet (Figure 10A; middle panel) with both phosphorylated forms attaining maximal levels within 10-30 min. Stat5b showed more protracted kinetics with serine phosphorylation peaking at 30-60 min and remained largely unchanged after 120 min in contrast to Stat5a which was significantly reduced at the same time point.

The main implication of this dissimilarity in phosphorylation for Stat5a/b is that they operate differently, explaining in part the lack of compensatory gene expression displayed in Stat5 single knockout mice. However, differences in immune-regulatory cells isolated from Stat5a or Stat5b null mice fail to display obvious cell defects [154]. Taken together, these findings suggest each transcription factor is redundant or may act to “fine-tune” T cell gene expression. Reconstitution assays employing the prolactin receptor and serine mutants of Stat5a (S726) and Stat5b (S731) failed to identify a significant loss in Stat5a/b transactivation potential [85]. However, reconstitution of the positionally conserved Stat1 (S727) mutant in functional relevant cells yielded definitive changes in gene transcription potential that could be correlated to association with minichromosome maintenance protein 5 [74].

The cellular location where Stat5a and Stat5b become serine phosphorylated is not clear, but it does not appear to be entirely dependent on an activated receptor complex since PMA alone could induce this event (Figure 12). These findings parallel earlier findings of phorbol-ester activation of Stat3 [76, 80]. The identity of the Stat5 proline-directed serine kinase is not immediately known. It has been shown that no membrane distal domains of the IL2Rβ were required to activate the kinase [144]. The only prerequisite for this event was a functional Jak3 since inactivation of this enzyme with Jak3 inhibitors or deletion of the Box1 domain of the IL2Rβ failed to promote Stat5a/b serine phosphorylation [50, 144]. We are left to conclude that the Stat5a/b serine kinase is not directly associated with TCGF receptor complexes but activated distal to the receptor.

Since T cells isolated from Stat5a/b-deficient mice fail to respond to the mitogenic effects of IL2 and likely other TCGFs [154], possibly due to reduced T cell expression of cdk-6 and cyclins A, D2, D3, and E, Stat5a/b probably regulates their transcription and therefore critical for cell cycle progression [1]. Based on these observations, one preliminary model that could be envisioned is that Sta5a/b represents a convergence point by which TCGFs drive T cell clonal expansion or protect against pro-apoptotic pathways. Indeed, given the diversity of the carboxyl termini shared between each Stat family member it seems plausible to expect that unique sets of genes could be controlled, in part, by distinct Stat proline-directed serine kinases. Indeed inhibitors to Mek, PI3K and mTor inhibited IL2-mediated Stat3 activation but did not affect Stat5 serine phosphorylation (Figure 13 and 14) or cell proliferation (Figure 19).

Based on studies presented herein and evidence by several other laboratories, an IL2 model depicted in Figure 25 can be conceived in which the IL2R comprised of three distinct single membrane spanning receptors direct the activation of Jak1 via the IL2Rβ and Jak3 by the γc. Activated Jaks phosphorylate key tyrosine residues on IL2Rβ, which serve as docking sites for downstream signaling molecules, including Mapk adapter protein Shc, Src enzymes as well as Stat5a/b [158]. Shc would subsequently recruit Grb-2/Sos activating the Ras/Erk pathway, and Grb-2/Gab-2, would engage the PI3K and mTor-signaling pathway, while the mitogenic signaling properties of Ras are linked to activation of the Erk signaling cascade driving phosphorylation of several transcription factors (e.g. Elk-1, Ets-1, Fos, and NF-AT) [72-74].

Our findings suggest that Mek1/2, PI3-K, mTor and Raf (A-, B-, and C-) pathways act to promote other cell processes and gene transcription events not linked to immediate cell survival as opposed to Stat5a/b which likely plays central role in acute cell survival and proliferative gene expression [13, 70].

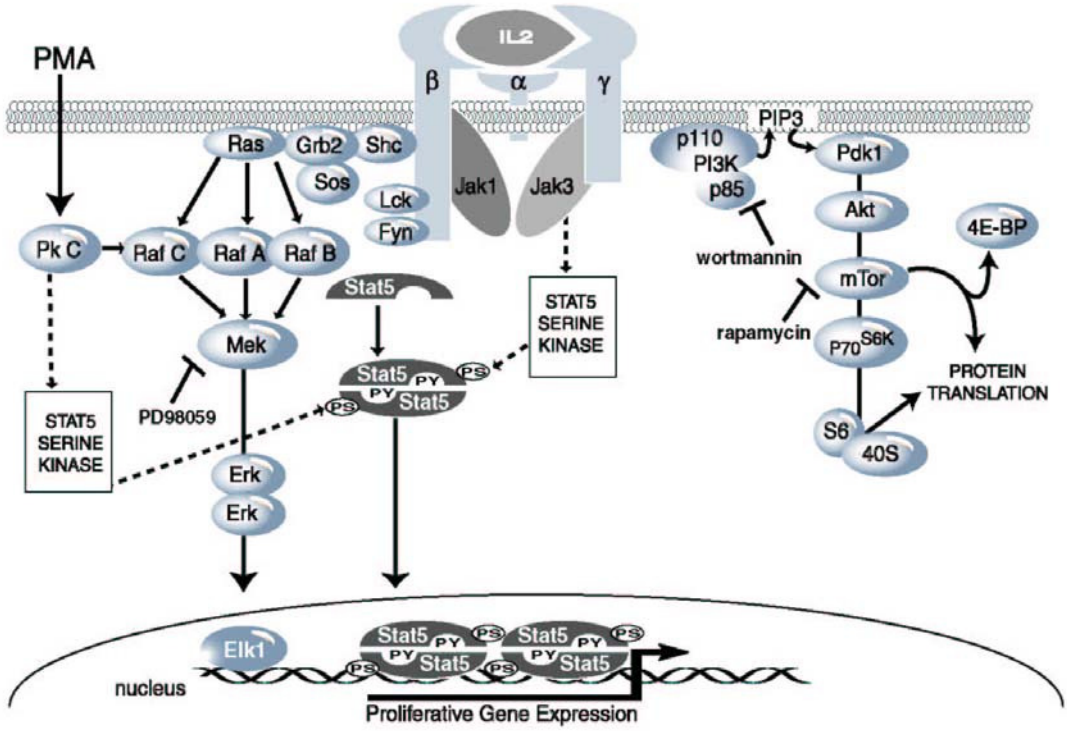


FIGURE 25. Schematic representation of IL2-mediated signaling pathways in human T cells. Engagement of the IL2 Receptor causes autophosphorylation and activation of Jak1 and Jak3 that induces at least three parallel signaling cascades; Ras-Raf-Mek, PI3K-Akt-mTor, and Stat5 serine kinase-Stat5a/b pathways. Tyrosine phosphorylation of Stat5a (Y699), Stat5b (Y701) and serine phosphorylation of Stat5a (S731) and Stat5b (S731) are dependent on Jak3, but not other multiple effector molecules (depicted) for tetrameric Stat5 DNA binding and transcription of proliferative genes in T cells.

Lastly, studies presented herein suggest that Stat5a and Stat5b serine phosphorylation are regulated differently than other Stats and support the conclusions by others that non-Stat5 regulatory serine kinase pathways are likely recruited by pro-inflammatory cytokines (e.g. IFNα/β, IFNγ, IL12) or regulatory cytokines (e.g. IL4, IL12,

IL13) to modulate cytokine release and cell differentiation, respectively. These findings have direct therapeutic consequences since inhibition of this putative proline-directed Stat5a/b serine kinase could be used to manipulate T cell expansion via G1-S cell cycle transition as found within graft versus host disease. Recent findings that Stat6 is also serine phosphorylated and void of a PSP or PXSP motif, suggest that its selective uncoupling might prove useful in combating Th2 diseases such as allergy or airway hypersensitivity [162].

Selective Disruption of Constitutively Active Stat3 by Antisense Oligonucleotides in a Human NK-like Tumor Cell Line

Lastly, we investigated whether constitutively active Stat3 provides a survival pathway in a lymphoid tumor cell line, suggesting that it alone could protect a cell against apoptosis, regardless of the activation state of PI3K, Mapk or Jak3.

We showed that:

(1) Stat3 is constitutively phosphorylated on the highly conserved Y701 and S727 residues (Figure 20A). IL2 was able to further induce phosphorylation of both residues (Figure 20A), most likely via Jak1 and Jak3, which are upstream tyrosine kinases of Stat3 in the IL2 signaling cascade.

(2) None of the Jak family members exhibit baseline enzyme activity in these cells (Figure 20B); therefore, we conclude that an independent pathway is responsible for the hyperactivity of Stat3.

(3) Antisense to Stat3 successfully disrupted its DNA binding activity but failed to affect Stat5 (Figure 21), as demonstrated with EMSA employing *m67* SIE and β-casein probes, respectively.

(4) Moreover, deletion of Stat3 protein promotes apoptotic cell death, as measured by the TUNEL assay (Figure 22).

(5) Culturing Stat3-depleted cells in the presence of physiological amounts (1nM) IL2 was able to rescue the cells from apoptosis (Figure 23).

(6) This event correlated with the hyperphosphorylation of Stat5a and b (Figure 24A), suggesting that the mechanism by which IL2 mediates the rescuing event is likely

via Stat5. Bcl-2 upregulation also correlated with Stat5 activity in Stat3-deleted cells (Figure 24B) suggesting a possible target gene regulated within this process.

Stat proteins are unique among signaling molecules in their ability to transmit signals from the cell surface to the nucleus and directly participate in the regulation of gene expression. Increasing evidence indicates that Stats signaling contributes to cancer formation and progression. Stat3 is known to be constitutively active in numerous malignancies, including leukemias, lymphomas, breast carcinoma, multiple myeloma, head and neck cancers, brain, lung and prostate cancers. Stat3 is thought to protect cells against apoptosis. Specific inhibition of Stat3 using dominant negative constructs or antisense molecules can induce apoptosis in different types of cancer cells [61, 64, 65, 163, 164]. The mechanism underlying cell survival supported by Stat3 has been linked to the transcriptional control of apoptotic regulatory genes, such as bcl-xL, bax [59] and mcl-1 [165]; as well as cell cycle regulator $p21^{wafl}$ [166]. Another interesting feature of Stat3 that it can also participate in apoptosis promoting cellular processes [167-169]. Chapman *et al.* developed a conditional Stat3 knockout and found that programmed cell death during mammary gland involution was significantly delayed [170]. The delay was proposed to be compensated by Stat1 and/or p53, with the former becoming active earlier in Stat3-deficient mammary cells [170].

Similarly, Stat5 activation is associated with survival of some leukemic cells and likely contributes to their resistance to undergo apoptosis by regulating bcl-xL [171, 172].
The paradoxical effects induced by Stat3 calls for further clarification of its role in certain types of malignant and normal cells. Possible redundant functions may contribute to the wide range of effects dictated by Stat family members, which should be the subject of future investigations.
Our results indicate that persistent Stat3 activity promotes cell survival in lymphoid cells. Furthermore, Stat5 can serve as a pro-survival factor mediated by IL2.

The role of alternate signaling pathways in maintaining lymphoid cell survival

As stated, IL2 activates a number of pathways including Jak1/3, Stat5a/b and 3, Mapk, PI3K and mTor. In summary, we studied the role of these pathways in lymphoid cells by blocking their activity with pharmacological inhibitors (PD98059 for Mek, wortmannin for PI3K, rapamycin for mTor, PNU156804 for Jak3) or disrupting their expression by using selective antisense oligonucleotides (γc, A-, B- and C-Raf, Stat3). We found that abrogation of γc expression leads to apoptotic death, likely by disrupting downstream signaling events. We confirmed these findings by using a novel inhibitor of Jak3, PNU156804, and showed that it disrupts the Jak3 target Stat5a/b and also Mapk activity. The finding that PNU156804 was able to block serine phosphorylation of Stat5 suggests that this serine kinase is dependent upon Jak3 activation. Several IL2-inducible serine kinase pathways seem to have the potential to mediate this posttranslational event. These pathways also have been implicated in the growth of lymphoid cells. PMA alone, a known Raf activator was able to induce serine phosphorylation of Stat5, findings that led us to investigate the role of these Raf family members (A-, B-, C-) in IL2 mediated cellular processes in lymphoid tumor and normal human T cells. Inactivation of PI3K or mTor had a limited effect on IL2 induced cell proliferation, while blocking Mek/Erk activity was somewhat more effective. Ultimately, however, each showed a limited inhibitory response on IL2-induced growth of two lymphoid derived cell lines, YT and CTLL2. Similar results were obtained with the application of selective antisense oligonucleotides targeted against A-, B- and C-Raf isoforms. The inhibition of these pathways failed to affect the serine phosphorylation of Stat5, a posttranslational modification required for full transcriptional activity. Blocking constitutively active Stat3 induced apoptosis. IL2 rescued the cells likely via activated Stat5. These findings led us conclude that Stats play a critical role in the growth and early survival of lymphoid cells (i.e. days).

What is the role of PI3K, Mapk and mTor? Likely they contribute to long-term cellular processes and survival. Our studies focused on relatively short kinetics 24-72 h. Studies to address this issue such as treating cells for several days to one week with Mek, mTor or PI3K inhibitors was not investigated. Likely such a treatment would have a deleterious effect on cell function and survival. Given the ubiquitous expression of these pathways,

long-term therapeutic strategies blocking their activity would likely prove too toxic to the patient. Our findings suggest that inhibition of acute Stats responses and their limited expression/function profile in non-hematopoietic cells, likely represent a more practical approach to regulating lymphoid derived diseases.

If the aforementioned conclusions are true then identification of Stat regulatory mechanisms such as kinases, phosphatases, nuclear transport proteins and their target genes will provide critical new targets and means for devising novel therapeutic strategies to control allergy, asthma, graft rejection, autoimmunity, and other debilitating diseases.

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X. APPENDIX

Publications this dissertation is based on:

1. Nagy, Z.S., Wang, Y., Erwin-Cohen, R.A., Wang, L., Aradi, J., Monia, B., Stepkowski S.M., Rui H., and Kirken, R.A. Interleukin-2 family cytokines stimulate phosphorylation of the Pro-Ser-Pro-motif of Stat5 transcription factors in human T cells: Resistance to suppression of multiple serine kinase pathways *The Journal of Leukocyte Biology*, in press, 2002 (IF: 4.342)

2. Stepkowski, S.M., Erwin-Cohen, R.A., Behbod, F., Wang, M-E., Qu, X., Tejpal, N., **Nagy, Z.S.**, Kahan, B.D., and Kirken, R.A. A Selective Inhibitor of Janus Tyrosine Kinase (Jak) 3, PNU156804, prolongs allograft survival and acts synergistically with cyclosporine but additively with rapamycin. *Blood* 2002 Jan 15; 99(2): 680-9 (IF: 8.977)

Other publications:

1. Stepkowski, S.M., **Nagy, Z.S.**, Kahan, B.D., and Kirken, R.A. The role of signals 1, 2 and 3 in induction of transplantation tolerance. *Transplant Proceedings.* 2001 Nov-Dec; 33(7-8): 3831-2 (IF: 0.678)

2. Stepkowski, S.M., Kirken, R.A. **Nagy, Z.S.**, Trawick, B.W., Wang, M., Tejpal, N., Wang, M., Tian, L., Clark, J., Kahan, B.D. The role of Stat5 in the induction of regulatory T cells in transplantation tolerance. *Transplant Proceedings.* 2001 Nov-Dec; 33(7-8): 3835-6 (IF: 0.678)

3. Stepkowski, S.M., Wang, M., Behbod, F., **Nagy, Z.S.**, Erwin-Cohen, R.A., Kahan, B.D. and Kirken, R.A. PNU156804 inhibits Jak3 tyrosine kinase and rat heart allograft rejection. *Transplant Proceedings.* 2001 Nov-Dec; 33(7-8): 3272-3 (IF: 0.678)

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Abstracts:

- **Nagy, Zs.**, Tőkés, Sz., Aradi, J. Studying the effect of antisense oligonucleotides targeted against Bcr-Abl, Bcl-2, c-Myb and $(dA)_{10}$ on the proliferation of K562

cells Annual Meeting of Hungarian Biochemical Society, 1999, Eger, Hungary, poster, in Hungarian

- **Nagy, Z.**, Stepkowski, S.S., Monia, B.P., and Kirken, R.A. „The role of Raf isoforms in mediating cell proliferation of lymphoid cells activated by multiple common IL2 receptor gamma chain cytokines". Seventeenth Annual Meeting on Oncogenes: Cancer Cell Signal Transduction, June 20-23, 2001, Hood College, Frederick, MD USA
- **Nagy, Zs.**, Kirken, R.A., Aradi, J. Studying the effect of antisense oligonucleotides targeted against IL2Rγ on the proliferation of lymphoid cells Annual Meeting of Hungarian Biochemical Society, 2002, Keszthely, Hungary, poster, in Hungarian
- **Nagy, Z.S.**, Aradi, J., Karras, J., and Kirken, R.A. Selective Inactivation of the IL2 Receptor Gamma Chain, and to a Lesser Extent, Constitutively Active Stat3 Induces Apoptosis in a Human NK Tumor Cell Line The Eighteenth Meeting on Oncogenes: The Lord of the Genes, June 21-24, 2002, The Salk Institute, La Jolla, CA, USA