Interleukin-7—Mediated Signaling and its Role in the Biology of T-Cell Acute Lymphoblastic Leukemia: Potential Targets for Therapeutic Intervention

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Para os meus pais e para a Ana

"Poderás dizer-me para que queres o barco, Para ir à procura da ilha desconhecida, Já não há ilhas desconhecidas. O mesmo me disse o rei, O que ele sabe de ilhas, aprendeu-o comigo, É estranho que tu, sendo homem do mar, me digas isso, que já não há ilhas desconhecidas, homem da terra sou eu, e não ignoro que todas as ilhas, mesmo as conhecidas, são desconhecidas enquanto não desembarcarmos nelas."

José Saramago *in* O Conto da Ilha Desconhecida

Summary

Acute lymphoblastic leukemia (ALL) is the most common form of leukemia and the most frequent malignancy in children. ALL results from the clonal expansion and accumulation of lymphoid cells arrested at different stages of differentiation. Patients with T lineage ALL (T-ALL) represent solely 12 to 18% of pediatric and 20 to 25% of adult ALL cases. However, until recent years they had a considerably poorer prognosis than B-lineage ALL patients. The use of risk-adjusted multi-agent intensive chemotherapy originated a remarkable improvement in treatment outcome, and recent reports indicate a very high remission rate and good overall survival. Nonetheless, a significant number of relapses still occurs in T-ALL and the intensive treatment regimens are often associated with long-term, severe complications that are particularly relevant in a disease with a high incidence of pediatric cases. Additionally, the response to conventional therapies and outcome of adults with T-ALL is considerably less favorable. Thus, novel therapeutic strategies are needed that specifically target the leukemic cells and minimize the detrimental side effects associated with conventional therapies. To achieve this goal, it is necessary to improve our knowledge regarding the biology of the leukemia cells.

Signal transduction pathways enable cells to respond adequately to their microenvironment, and hence are fundamental for the establishment of an equilibrium between cell division, differentiation and apoptosis. Disruption of the signaling machinery by leukemogenic mutations subverts the homeostatic balance and consents the expansion of the malignant clone beyond the limits imposed upon non-transformed cells. Moreover, the leukemic cells are not oblivious to their surrounding environment and external factors such as cytokines might have a positive or negative regulatory role over the development of the disease. The present work focuses on IL-7-mediated signal transduction and its effect on survival and cell cycle progression of T-ALL cells, with two main objectives: 1) to evaluate the possible role of this cytokine in the pathophysiology of T-ALL; and 2) to try and identify putative molecular targets for the development of specific therapeutic agents.

IL-7 belongs to a group of cytokines that also include IL-2, IL-4, IL-9 and IL-15, whose receptors share the common gamma chain (γ_c). They have distinct effects on T cell development, survival, proliferation and differentiation, and have been directly or indirectly associated with T cell malignancies. Therefore, we first analyzed the effect of γ_c-signaling cytokines on the proliferation of T-ALL cells (Chapter 2). We demonstrate that primary T-ALL cells proliferate in response to all γ_c-signaling cytokines. IL-9 and IL-15 are implicated for the first time as potentially involved in the biology of the disease. Importantly, we show that IL-7 is the γ_c-cytokine that mediates the most striking proliferative effect. In Chapter 3, we demonstrate that IL-7-mediated proliferation is the result of both increased viability and cell cycle progression of T-ALL cells. We show that Bcl-2 protein expression is mandatory for IL-7-mediated viability, and that downregulation of $p27^{kip1}$ cyclin-dependent kinase inhibitor is critical for both cell cycle progression and Bcl-2 upregulation induced by IL-7. Our results concur with the notion that $p27^{kip1}$ may function as a tumor suppressor gene, not only because it is a negative regulator of cell cycle progression but also due to its association with induction of apoptosis of primary malignant cells.

Most of our studies were performed using primary T-ALL cells. Despite being considerably more laborious and time-consuming than using immortalized cell lines it is our conviction that this approach reflects more accurately the biology of the leukemia. However, to overcome the technical limitations associated with insufficient availability of viable primary cells, we established a novel leukemia cell line derived from a T-ALL patient, which has the advantage of being IL-7-dependent and maintaining the phenotype and functional characteristics of the parental primary leukemia cells. This cell line was named TAIL7 and is described in Chapter 4. We demonstrate that TAIL7 is tumorigenic in NOD/SCID mice with a pattern of in vivo expansion that resembles that of primary cells rather than other T-ALL cell lines. Thus, TAIL7 can become a valuable tool for the study of cytokine-mediated signaling pathways, identification of novel targets and screening of specific inhibitors for therapeutic intervention in T-ALL. In Chapter 5, we use both TAIL7 and primary cells to identify which pathways are fundamental for IL-7-mediated cell expansion. By using pharmacological inhibitors of specific signaling pathways, we demonstrate that PI3K/Akt(PKB) pathway is indispensable for $p27^{kip1}$

downregulation, Bcl-2 upregulation, and consequent cell cycle progression and increased survival. We further show that PI3K is necessary for CD71 expression and T-ALL cell activation.

Overall, our studies establish the importance of IL-7 as an *in vitro* growth factor for T-ALL and provide several putative targets for novel therapies against this malignancy.

Résumé

La leucémie lymphoblastique aiguë (LLA) est la forme la plus commune de leucémie et le cancer le plus fréquent chez les enfants. La LLA résulte de l'expansion clonale et de l'accumulation des cellules lymphoïdes arrêtées à différentes étapes de différenciation. Les patients présentant la leucémie de lignée T (LLA-T) représentent seulement 12 à 18% des leucémies pédiatriques et 20 à 25% des leucémies de l'adulte. Cependant, jusqu'à récemment, les patients de LLA-T ont un prognostique considérablement plus faible que ceux atteints de leucémies B. L'utilisation de la chimiothérapie intensive, risque-ajuéste et aux agents multiples, a provoqué une amélioration sensible des résultats de traitement, et les rapports récents indiquent un taux très élevé de rémission et une bonne survie générale. Néanmoins, un nombre significatif de rechutes se produit toujours pour les LLA-T et les régimes de traitement intensifs sont souvent associés à des complications graves et à long terme, qui sont particulièrement importantes dans une maladie avec une incidence élevée de cas pédiatriques. De plus, la réponse aux thérapies conventionnelles et la survie des adultes présentant des LLA-T est considérablement moins favorable. Ainsi, de nouvelles stratégies thérapeutiques sont nécessaires afin de cibler spécifiquement les cellules leucémiques et de réduire au minimum les effets secondaires négatifs associés aux thérapies conventionnelles. Afin d'atteindre cet objectif, il est nécessaire d'améliorer la notre compréhension de la biologie des cellules leucémiques.

Les voies de transduction de signal permettent aux cellules de répondre en juste proportion à leur micro-environnement, et par conséquent sont fondamentales à l'établissement d'un équilibre entre la division cellulaire, la différenciation et l'apoptose.

L'altération des mécanismes de signalisation par des mutations oncogèniques perturbe l'équilibre homéostatique et permet l'expansion du clone tumoral au delà des limites imposées aux cellules non-transformées. Par ailleurs, les cellules leucémiques ne sont pas étrangères à leur microenvironnement et des facteurs externes tels que des cytokines pourraient avoir un rôle de régulation positif ou négatif sur le développement de la maladie. La présente etude se concentre sur le transduction de signal induit par l'IL-7 et son effet sur la survie et la progression du cycle cellulaire des cellules leucémiques, avec deux objectifs principaux: 1) évaluer le rôle possible de l'IL-7 dans la pathophysiologie des leucémies, et; 2) identifier des cibles moléculaires potentielles pour le développement d'agents thérapeutiques spécifiques.

L'IL-7 appartient à un groupe de cytokinesqui incluent également l'IL-2, IL-4, IL-9 et IL-15, dont les récepteurs partagent la chaîne gamma commune (γ_c). Ces cytokines ont des effets distincts sur le développement, la survie, la prolifération et la différenciation des cellules T, et ont été directement ou indirectement associées aux tumeurs de cellules T. Par conséquent, nous avons tout d'abord analysé l'effet des cytokines utilisant la γc sur la prolifération des cellules leucémiques (Chapitre 2). Nous démontrons que les cellules leucémiques primaires prolifèrent en réponse à toutes les cytokines qui signalisent par la γc. L'IL-9 et l'IL-15 sont ici décrites pour la première fois comme potentiellement impliquées dans la biologie des LLA-T. De façon notable, nous prouvons qu'IL-7 est la citokine signalant par la γc qui induit l'effet proliératif le plus important. Dans le Chapitre 3, nous démontrons que la prolifération induite par l'IL-7 résulte d'une plus grande viabilité et de la progression du cycle cellulaire des cellules LLA-T. Nous montrons également que l'expression de la protéine Bcl-2 est requise pour

la viabilité induit par l'IL-7, et que la régulation négative de l'inhibiteur des 'cyclin-dépendant' kinases $p27^{kip1}$ est essentiel à la progression du cycle cellulaire et à la régulation positive de Bcl-2 induite par l'IL-7. Nos résultats renforcent l'hypothèse que $p27^{kip1}$ peut fonctionner comme un gène suppresseur de tumeur, non seulement parce que c'est un régulateur négatif de la progression du cycle cellulaire mais également en raison de son association avec l'induction de l'apoptose des cellules leucémiques primaires.

La plupart de nos études ont été réalisées en utilisant les cellules LLA-T primaires. Bien qu'étant plus laborieux et beaucoup plus long que d'utiliser des lignées de cellules immortalisées, nous sommes convaincus que cette approche reflète plus prècisement la biologie de la LLA-T. Cependant, pour surmonter les limitations techniques dues à une disponibilité insuffisante de cellules primaires leucémiques viables, nous avons établi une nouvelle lignée de cellules de LLA-T dérivée d'un jeune patient, qui a l'avantage d'être dépendante de l'IL-7 et de maintenir le phénotype et les caractéristiques fonctionnelles des cellules LLA-T parentales. Cette lignée de cellules a été appelée TAIL7 et est décrite dans le chapitre 4. Nous démontrons que TAIL7 est tumorigènique chez des souris NOD/SCID avec un profil d'expansion in vivo qui semblable à celui des cellules leucémiques primaires plutôt qu'à celui d'autres lignées de cellules LLA-T. Ainsi, TAIL7 peut devenir un outil précieux pour l'étude des voies de signalisation induites par les cytokines, pour l'identification de nouvelles cibles moléculaires et pour l'évaluation d'inhibiteurs spécifiques pour le traitement de LLA-T. Dans le chapitre 5, nous utilisons TAIL7 et des cellules leucémiques primaires afin d'identifier quelles voies sont fondamentales pour l'expansion de LLA-T induite par l'IL-7. Nous démontrons à l'aide d'inhibiteurs pharmacologiques spécifiques des voies de

transduction de signal, que la voie PI3K/Akt(PKB) est indispensable à la régulation négative de $p27^{kip1}$, à la régulation positive de Bcl-2, et à la progression du cycle cellulaire et l'augmentation de la survie. Nous montrons également que PI3K est nécessaire à l'activation de cellules LLA-T et à l'expression de CD71.

De façon générale, nos études établissent l'importance de l'IL-7 comme un facteur de croissance pour LLA-T et présentent plusieurs cibles potentielles pour de nouvelles thérapies pour le traitement de ce cancer.

Resumo

A leucemia linfoblástica aguda (LLA) é a forma mais comum de leucemia e a neoplasia mais frequente em crianças. A LLA resulta da expansão clonal e acumulação de células linfóides devido ao bloqueio do processo normal de diferenciação celular. A leucemia linfoblástica aguda de células T (LLA-T) representa 12 a 18% dos casos de LLA em crianças e 20 a 25% dos casos adultos. Até há pouco tempo, os doentes com LLA-T apresentavam um prognóstico consideravelmente pior que os doentes com LLA de linhagem B. A utilização combinada e intensiva de vários agentes quimioterápicos, adaptada aograu de risco de cada doente, permitiu progressos consideráveis no tratamento da doença, resultando numa melhoria significativa das taxas de remissão e de sobrevivência. Não obstante, um número significativo de recidivas continua a ocorrer, e os regimes terapêuticos actualmente aplicados estão, com frequência, associados a complicações severas a longo prazo, o que é particularmente relevante numa doença que atinge preferencialmente as crianças. Por outro lado, a resposta à terapêutica convencional é substancialmente pior nos adultos. Assim sendo, são necessárias novas estratégias terapêuticas que atinjam selectivamente as células leucémicas, aumentando a curabilidade da doença e minimizando os efeitos secundários actualmente observados. Para alcançar tal objectivo será necessário conhecer melhor a biologia das células T leucémicas.

As cascatas de transdução de sinal permitem que uma célula responda adequadamente ao seu micro-ambiente, e ,portanto, são fundamentais para o estabelecimento de um equilíbrio entre divisão celular, diferenciação e apoptose. Mutações oncogénicas que perturbem a maquinaria molecular envolvida nas cascatas de transdução de sinal rompem a homeostasia e permitem a expansão do clone maligno à revelia dos limites impostos às células normais. Por outro lado, as células leucémicas não estão isoladas do meio que as rodeia, e factores externos, como as citoquinas, poderão ter um papel regulador, positivo ou negativo, na progressão da doença.

A presente tese debruça-se sobre as cascatas de transdução de sinal estimuladas pela interleucina 7 (IL-7) e os seus efeitos na sobrevivência e progressão no ciclo celular das células T leucémicas, com dois objectivos subjacentes: 1) avaliar o possível papel da

IL-7 na fisiopatologia da LLA-T; e 2) tentar identificar possíveis alvos moleculares para o desenvolvimento de agentes terapêuticos específicos.

IL-7 pertence a um grupo de citoquinas que inclui a IL-2, a IL-4, a IL-9 e a IL-15 ("citoquinas-γ_c") cujos receptores partilham uma subunidade, denominada "cadeia gama comum" (γ_c). Estas citoquinas têm efeitos distintos a nível do desenvolvimento, sobrevivência, proliferação e diferenciação dos linfócitos T, e têm sido directa ou indirectamente associadas a tumores desta linhagem de linfócitos. Em consequência, começámos por analisar o efeito das citoquinas-γ_c na proliferação de células LLA-T, e observámos (Capítulo 2) que as células primárias LLA-T proliferam em resposta a todas as citoquinas-γ_c. As citoquinas IL-9 e IL-15 são pela primeira vez descritas como podendo estar potencialmente envolvidas na biologia da doença. Verificámos ainda que a IL-7 é a "citoquina-γ_c" que promove uma resposta proliferativa leucémica mais potente. Este dado, de particular importância, levou-nos a procurar esclarecer o papel desta citoquina na biologia da LLA-T. No Capítulo 3, demonstramos que a IL-7 promove a proliferação das células LLA-T através do aumento de viabilidade e progressão no ciclo celular. O aumento de viabilidade estimulado por IL-7 resulta de um aumento dos níveis de expressão da proteina anti-apoptótica Bcl-2 e é necessariamente mediado por esta última. Para além disso, tanto o aumento da expressão de Bcl-2, como a progressão no ciclo celular, induzidos por IL-7, resultam do decréscimo de expressão do inibidor do ciclo celular, $p27^{kip1}$. Os nossos resultados suportam a noção de que $p27^{kip1}$ poderá funcionar como um gene supressor tumoral, não apenas porque é um regulador negativo da progressão no ciclo celular, mas igualmente devido ao facto de estimular a apoptose das células malignas.

A maioria dos nossos estudos foi efectuada utilizando células leucémicas primárias. Apesar dos estudos com células primárias serem consideravelmente mais laboriosos do que aqueles que fazem uso de linhas celulares imortalizadas, é nossa convicção de que os primeiros reflectem mais apropriadamente a biologia da leucemia. Contudo, por forma a ultrapassar as limitações técnicas associadas ao número insuficiente de células primárias viáveis para realizar determinado tipo de experiências, estabelecemos uma nova linha celular a partir de uma amostra de sangue periférico de um doente com LLA-T. Esta linha celular, descrita no Capítulo 4 e designada TAIL7, tem a

vantagem de depender de IL-7 para a sua sobreviência e expansão, e de manter as características fenotípicas e funcionais das células primárias progenitoras. As células TAIL7 são tumorigénicas em ratinhos NOD/SCID, com um padrão de expansão *in vivo* similar ao de células primárias e não ao de outras linhas celulares LLA-T. Como tal, a linha TAIL7 poderá tornar-se uma ferramenta valiosa no estudo da transdução de sinal estimulada por citoquinas, identificação de potenciais alvos terapêuticos e "screening" de inibidores específicos para esses alvos. No Capítulo 5, usamos tanto a linha TAIL7 como células primárias para identificar as cascatas de transdução de sinal fundamentais para a expansão celular promovida por IL-7. Através do uso de inibidores farmacológicos, demonstramos que a cascata de transdução de sinal PI3K/Akt(PKB) é indispensável para a diminuição de expressão de $p27^{kip1}$, aumento de Bcl-2, e consequente progressão no ciclo celular e aumento de viabilidade. Para além disso, a activação das células LLA-T e o concomitante aumento de expressão do marcador de activação CD71, estimulados por IL-7, são também dependentes de PI3K.

Os nossos estudos demonstram a importância da IL-7 como factor de crescimento de LLA-T *in vitro*, e oferecem vários alvos possíveis para o desenvolvimento de novas terapias contra este tipo de tumores hematológicos.

CONTENTS

Chapter 1

GENERAL INTRODUCTION

"At each point in the history of leukemia there have been instances of lost time and opportunity because of unreasoned resistance to innovation"

Donald Pinkel *in* Chilhood Leukemias

General Introduction Contents

Leukemia And T-ALL: Old Diseases New Challenges

Brief Historical Overview

More than 150 years have passed since leukemia was first recognized as a distinct disease. In 1845, the German doctor Rudolph Virchow identified this new entity, which he named "weisses blut", white blood, because of the enormous accumulation of white cells found in a patient with a far-advanced chronic disease [1]. Two years later he introduced a new term with the same meaning — the greek-derived word "leukämie". The development of staining methods in 1891 allowed for the distinction between the different leukocyte populations and concomitant identification of leukemia cell types (Ref. [2] and citations therein). Previously, in 1857, N. Friedrich had described for the first time acute leukemia [3]. Broadly, leukemia is classified based on the distinction between acute *versus* chronic and lymphoid *versus* myeloid. Initially, acute and chronic referred to the relative time-span of survival of patients when effective therapy was not available. The improvements in treatment efficacy redefined these terms, and presently "acute" is associated with leukemia characterized by rapid proliferation of blast cells, whereas "chronic" refers to leukemia with slower proliferation of malignant cells that are in general relatively well differentiated [4].

Throughout the 20th Century the development of new technologies, such as electron microscopy, karyotyping, immunophenotyping and molecular genotyping, together with the accumulation of empirical clinical data, led to significant progresses in the characterization of leukemias. The realization that apparently similar types responded differently to the same kind of treatment created the need to use the new technologies to fine-tune the classification of leukemias beyond strictly morphological criteria, in order to clearly and accurately distinguish them.

The discovery in 1960 of the chronic myeloid leukemia-associated Philadelphia Chromosome [5], a minute chromosome that was later shown to result from a t(9;22) translocation [6], was the first step towards the identification of many non-random chromosomal aberrations associated with specific types of leukemia. Chromosomal analysis, DNA probing, fluorescent in situ hybridization, polymerase chain reaction and microarray analysis are relevant examples of technologies that have been applied to allow

the molecular definition of leukemias. Furthermore, the use of molecular biology techniques with the purpose of classifying leukemias resulted in the discovery of many new genes, including oncogenes and tumor suppressors, which are critical for proliferation, survival and differentiation [7-10].

In 1973, Borella and Sen found that the leukemic cells present in some children with acute lymphoblastic leukemia (ALL) presented an immunophenotype characteristic of immature T-cells, indicating their thymic origin [11]. Importantly, they initiated the classification of leukemia by biological function when they further demonstrated that T-ALL was clinically and biologically distinct from B-ALL [12]. Immunophenotyping not only contributed to the clarification of the enormous biologic heterogeneity of leukemia but also became a precious tool in the study of human hemato- and lymphopoiesis.

Acute Lymphoblastic Leukemia

ALL is a heterogeneous disease with variations in morphological, cytogenetic and immunological features [13], resulting from the clonal expansion and accumulation of lymphoid cells arrested at different stages of differentiation [14]. The malignant clone may be of B- or T-cell origin, and the leukemia is thought to arise in the bone marrow or thymus. ALL is the most frequent childhood malignancy in the USA, accounting for 25% of all pediatric cancers [15] and 75 to 80% of pediatric leukemias [4]. ALL has its greatest incidence in children less than 10 years old, with a second frequency peak beginning around the age 50 [16].

Most of the symptoms and signs associated with ALL at diagnosis derive from the collapse of normal hematopoiesis. The most common manifestations, weakness, excessive tiredness, pallor and lassitude are nonspecific manifestations of developing anemia. Bleeding, due to a significant reduction of platelet levels, occurs in about half of the patients. Less usually, neutropenia may lead to predisposition to bacterial infections, and fever may thus result. Pain, in particular bone-associated, occurs in around 25% of ALL patients. Less common complaints, present in fewer than 10% of the patients and more associated with T-cell ALL, are enlarged lymph nodes or headache and vomiting resulting from increased intracranial pressure due to meningeal involvement with leukemia [15].

The first attempts to sub-classify ALL were naturally based on morphologic and cytochemical features. Various cytochemical stains, like acid phosphatase or myeloperoxidase, have been used to rule out or delineate some leukemic types. However, the information obtained by these methods to subdivide ALL has limited practical value. Because leukemic cells are morphologically indistinguishable from normal immature lymphoid cells, attempts to define relevant sub-groups have also been largely unsuccessful [17]. Nonetheless, one classification system devised by the French-American-British (FAB) Cooperative Working Group became broadly accepted [18]. Three subtypes were defined: L1, in which blasts are small with scanty cytoplasm and indistinct nucleoli; L2, in which blasts are larger with increased amounts of cytoplasm, irregular nuclear membranes and prominent nucleoli; and L3, in which blasts have basophilic cytoplasm with vacuolization. Around 85-95% of childhood ALL are FAB L1, 5-15% L2 and only 1% or less L3. Adult ALL has a higher incidence of L2 subtype [19]. In practice, the morphologic features of ALL tend to form a continuum between L1 and L2, and neither subtype has been shown to have any significant immunophenotypic, genetic or clinical correlate [4]. Thus, although recognition of the morphologic characteristics of leukemic lymphoblasts can be important in avoiding misdiagnoses, this approach to ALL classification has been largely supplanted by analysis of genotype and immunophenotype [20].

Specific cytogenetic lesions in ALL can be correlated with immunophenotype [21], but generally not with morphologic subtypes [22]. Numeric or structural chromosomal changes often occur in leukemia. According to the modal number of chromosomes, ALL can be classified into five major groups [23]. The karyotype may be diploid, i.e. normal (46 chromosomes with no evident structural abnormalities; 10-15% of cases); pseudodiploid (46 chromosomes with structural abnormalities, most frequently translocations; around 40% of cases); hyperdiploid with 47 to 50 chromosomes (10-15% of cases); hyperdiploid with more than 50 chromosomes (25-30% of cases); and hypodiploid (less than 8% of cases). The integration of karyotypic analysis into clinical studies has proven of great usefulness in predicting clinical outcome and devise risk-adjusted therapy [24-27]. For example, patients with hyperdiploidy >50 have more favorable presenting features, significantly better prognosis and higher cure rates than other groups [25-27]. Importantly,

the ability to characterize cases of ALL not only by numeric but also through structural chromosomal abnormalities further advanced our understanding of leukemia pathobiology and increased the capacity to formulate risk-adapted therapy [23,28]. For instance, the Philadelphia chromosome, or t(9;22), occurs in about 4% of children with ALL and was identified as an adverse prognostic factor [29,30]. Moreover, many other non-random structural lesions, mainly translocations, have been found both in B- and T-ALL and shown to result in oncogenic fusion proteins that affect normal cell function by deregulating signal transduction pathways critical for cell differentiation and proliferation (reviewed in [23,31]).

Based on the reactivity towards a panel of lineage-associated antibodies, ALL can be broadly classified as being of B- or T-cell origin, and subsequently divided according to the stage of differentiation. The classification of leukemia cells according to their stage of maturation is of broad importance for a better understanding of the biology of ALL and for the assessment of treatment outcome in relation to maturational stage for prognostic reasons. The advent of monoclonal antibodies permitted an increasing gathering of information regarding human lymphocyte maturation. Consequently, diverse classifications have been proposed based on the expression of several differentiation-associated antigens [32-36]. However, despite many workshops on the topic, no consensus has yet been achieved as to the most accurate or clinically significant immunological classification to be used. An example of an early immunophenotypic classification considered three ALL subtypes: common (expressing CD10, a marker initially thought to be exclusive of B lineage ALL), T (expressing T-cell markers) and "null" (for which no B- or T-cell antigen expression was detected) [36]. Other groups considered that the "non-T-ALL" subtypes were mostly of B-cell origin and proposed the subdivision of B-cell ALL according to apparent maturational stage [37]. As new differentiation markers were discovered, it became apparent that most, if not all, ALL cases could in fact be defined as having a B or T-cell-related phenotype, and that a considerable number of T-ALL expressed the "B-cell ALL" CD10 antigen. Consequently, more refined and clinically useful classifications emerged, which defined four ALL subtypes: early pre-B, pre-B, B and T [38]. Presently, most investigators also subdivide T-ALL and consider leukemic T lymphoblasts as resulting from thymocytes arrested in one of three [17] or four [4] maturation

stages. The ultimate clinical significance of these subclassification schemes remains to be established, but several reports indicate that patients with more immature T-ALL achieve remission less often [34] and have a worse prognosis [39,40] than more differentiated subtypes.

In this thesis, we adopted the criteria of Uckun and colleagues [40] and the European Group for Immunological Characterization of Leukemias (EGIL) [41], which are somewhat complementary. The first defines three stages of maturation: pro-thymocytes ($CD7^+$ only), immature thymocytes ($CD7^+$, CD2 and/or $CD5^+$, $CD3^-$) and mature thymocytes ($CD7^+$, $CD2^+$, $CD5^+$, $CD3^+$). The latter use a further marker, CD1, and consider the following stages: pro-T-ALL ($CD7^+$ only), pre-T-ALL ($CD7^+$, $CD1^-$, CD2 and/or CD5 and/or $CD8^+$, $CD3^-$), cortical T-ALL ($CD1^+$, independently of the presence of other markers) and mature T-ALL ($CD1^-$, $CD3^+$).

T-cell Acute Lymphoblastic Leukemia

Approximately 12 to 18% of children [42] and 20 to 25% of adult [19] ALL cases have leukemia of T-cell phenotype (T-ALL), which is associated with higher initial white blood cell (WBC) counts (frequently higher than 50,000/μL), mediastinal masses, male gender (20-30% more frequent in males), involvement of the central nervous system, and enlargement of the spleen, liver and lymph nodes [13,43-46]. Until recent years T-ALL patients, were reported to have a poorer prognosis than B-lineage ALL patients [46-48]. However, the use of risk-adjusted multi-agent intensive chemotherapy has led to a remarkable improvement in treatment outcome, and recent reports indicate a very high remission rate and good overall survival, similar or even better than for B-ALL, both in children and adults [39,49-51]. Currently more than 70% of children with ALL, either B or T, are alive and disease-free at 5 years [31]. Despite these successes, a significant number of relapses still occurs in T-ALL and the intensive regimens are often associated with long-term, severe complications that include the development of secondary tumors, growth retardation, cardiac and endocrine dysfunction and neuropsychological complications [17,31], which are of great relevance in a disease that has a high pediatric incidence. Further strengthening of the currently used intensive chemotherapeutic protocols will likely result in increased risk of adverse effects without significantly augmenting treatment efficacy. Therefore, a new challenge presently arises to develop novel, more efficient therapeutic

strategies that specifically target the leukemic cells and consequently minimize or, ideally, abrogate the detrimental side effects associated with conventional therapies. To achieve this goal, it will certainly be essential to continue to improve our knowledge regarding the causation of leukemia and the biological regulation of leukemic cells.

At present, despite years of research, the ultimate causation of leukemia remains poorly understood. Four historical approaches have been followed to try and explain the origin of leukemia: infectious [52], genetic [53], physical [54] and chemical (reviewed in [2]). Despite much controversy regarding which ones are actually relevant for the disease, it should not be excluded that all of them may be relevant under particular circumstances. In fact, despite the several distinct conditions and agents that augment the incidence of leukemia, no single event appears to be sufficient to either explain all cases of the disease or even induce *per se* the malignancy in an individual [55]. It rather seems that for T-ALL to arise there must be a concatenation of factors involving alterations in the genome, gene expression, signal transduction pathways, tumor microenvironment, and the capacity of the immune system to recognize and respond to the neoplastic cells.

Genetic And Signaling Aberrations In T-ALL

Homeodomain and bHLH Transcription Factors

Extensive cytogenetic studies during the recent years have indicated a distinct pattern of nonrandom karyotypic aberrations in T-ALL. Rearrangements involving the proximal bands of chromosone 14 (14q11) and two regions of chromosome 7 (7q34 to q36 and 7p15) appear to be particularly frequent in T-cell malignancies [23]. T-cell-specific abnormalities most frequently involve 14q11. The first recurring abnormality to be defined was the reciprocal translocation between chromosomes 8 and 14, t(8;14)(q24;11) [56]. Subsequently, other abnormalities involving 14q11 were recognized. So far, chromosome 7 is known to be affected by two recurring abnormalities that involve 7p15 and eight that involve the distal long arm [23]. Nonrandom translocations involving chromosome 14 and chromosome 7 are associated with transcriptionally active sites of the TCRα/δ (on chromosome 14) and TCRβ (on chromosome 7) loci, which are located at the breakpoints of these abnormalities [57]. These translocations probably result from illegitimate V(D)J recombination during the process of generating functional T-cell

receptors [58-60], and lead to the abnormal activation of several transcription factor genes, including members of the bHLH (MYC, TAL1, TAL2, LYL1, BHLHB1), LIM (LMO1, LMO2) and homeodomain (HOX11, HOX11L2) families (reviewed in [23,61]).

TAL1, normally involved in erythroid differentiation and in the earliest steps of hematopoietic lineage commitment, is overexpressed in as much as 65 % of T-ALL cases [62]. This abnormal expression results from either t(1;14), from local DNA rearrangements or from ectopic expression without any obvious alteration of the TAL1 gene [63]. The translocations associated with TAL1-related genes TAL2, LYL1 and the recently described BHLHB1, are rarely observed. In a recent study however, increased expression of LYL1 was observed in 20% of T-ALL cases and found to be independent of cytogenetic abnormalities affecting the LYL1 locus [53]. Myc overexpression, resulting from t(8;14) occurs in about 2% of T-ALL cases [56].

Overexpression of the cysteine-rich LIM-only proteins LMO1 and LMO2, which are not normally expressed in lymphoid cells, results from t(11;14) and can account for up to 7% of childhood T-ALL cases using conventional cytogenetics [64]. Interestingly, TAL1 aberrant expression is often accompanied by overexpression of LMO proteins [53,65], and TAL1 and LMO are highly synergistic inducing T-cell tumorigenesis in mice [66]. In fact, LMO proteins form heterocomplexes and act in concert with TAL1 in T-ALL [67].

HOX11 is a homeobox gene critical for splenic development that is activated in some T-ALL as a result of t(10;14) or t(7;10). HOX11 overexpression has been observed in up to 7% of T-ALL cases [57]. The importance of HOX11 for leukemogenesis was underscored by the fact that transgenic mice overexpressing HOX11 in the thymus develop T-cell malignancies [68]. Recently, another homeobox gene HOX11L2 was found to be a relevant target for T-ALL leukemogenesis [69].

Modulation Of Oncogenic Transcription Factors By External Stimuli

The transcription factors mentioned above are regulated by external signals under normal circumstances and are probably exogenously modulated even when overactivated or misexpressed in the context of malignant transformation. TAL1 and TAL2 are phosphorylated at specific serine residues by the mitogen activated protein kinase ERK1 and the cAMP-dependent protein kinase PKA, consequently altering their DNA binding

activities [70-72]. During erythroid differentiation, erythropoietin (Epo) induces phosphorylation of TAL1 [73], and TAL1 levels are upregulated by Steel factor and Epo [73,74]. Importantly, TAL1 leukemogenic effect can be dramatically accelerated by casein kinase IIα, a serine/threonine kinase for which TAL1 is a substrate, by transgenic co-expression in mice [75], suggesting that hyperphosphorylation of inappropriately expressed TAL1 is highly oncogenic [57]. These studies clearly indicate that the functional properties of TAL polypeptides can be modulated by several distinct extracellular stimuli. The interaction of LYL1 with NF-κB1, a downstream target of several pathways [76]; the upregulation of HOX11 mRNA by the mitogen phytohemagglutinin (PHA) together with the evidence that tyrosine phosphorylation is required for this upregulation [77]; and the upregulation of Myc by MAPK, PI3K and PKC pathways [78,79] are further examples that support the notion of an interaction between the T-ALL-associated transcription factors and signal transduction networks. Also noteworthy is the fact that HOX11 interacts with the protein phosphatases PP1 and PP2A and thereby accelerates cell cycle progression [80]. In turn, HOX11 appears to be regulated in a cell cycle-dependent manner, particularly in the G1/S phase boundary [81], when extracellular stimuli regulate the progression of the cycle.

Overall, these studies provide strong evidence of the probable impact of extracellular signals, such as those associated with cytokines or growth factors, in modulating and hence participating in the leukemogenic process. Direct evidence of the critical importance of signal transduction pathways for T-ALL development results from additional nonrandom translocations and deletions associated with this malignancy.

Signaling Molecules Implicated In T-ALL

Between 3 and 5% of T-ALL patients have a t(7;9) translocation between the TCRβ locus and TAN-1, the human homologue of Drosophila Notch gene [82], a molecule that functions in signal transduction pathways involved in cell-fate determination during development, and which has been shown to play an important role in T-cell lineage commitment [83-85]. Notch has been further implicated in T-cell leukemogenesis, since Notch3 transgenic mice develop early and aggressive T-cell neoplasias [86]. In addition, mice overexpressing the Notch ligand delta-4 develop T-cell malignancies [87].

Overexpression of the Src family protein tyrosine kinase gene LCK in T-ALL results from t(1;7) [88,89]. Notably, LCK transgenic mice develop T-cell maligancies [90], suggesting a role for deregulated LCK expression in T-cell oncogenesis.

Another chromosomal translocation, t(9;12), found in a patient with T-ALL was shown to fuse the 3' portion of Jak2, a gene involved in the cytokine-induced Jak/STAT pathway, to the 5' region of TEL, a gene encoding a member of the ETS transcription factor family [91]. The resulting fusion protein has constitutive Jak2 tyrosine kinase activity, confering cytokine-independent proliferation to the IL-3-dependent Ba/F3 cell line. Furthermore, TEL-Jak2 transgenic mice develop fatal T-cell leukemia [92], and the leukemic cells show expression of the tyrosine phosphorylated fusion protein and activation of the downstream targets STAT1 and STAT5. The latter was reported to be essential for the disease induced by TEL-Jak2 [93]. Importantly, STAT1 and STAT5 constitutive activation was found in the peripheral blood cells of patients with ALL [94].

Expression of the anti-apoptotic oncoprotein Bcl-2 seems to show marked variation amongst childhood ALL patients [95]. Nonetheless, high Bcl-2 expression in primary T-ALL cells is predictive of slow early response to therapy [95], and IL-7-mediated protection from *in vitro* spontaneous apoptosis correlates with Bcl-2 upregulation [96].

Tumor suppressor gene deletion or inactivation is also associated with T-ALL. The Rb gene, which was initially identified because of its inactivation in retinoblastoma, is inactivated in some cases of T-ALL [13,97]. Other cell cycle regulators have similarly been implicated in T-ALL, particularly the cyclin-dependent kinase (cdk) inhibitor, $p16^{ink4a}$. Several groups have reported $p16^{ink4a}$ lesions, particularly homozygous deletions, to occur very frequently in T-ALL [98-99]. Hypermethylation has further been identified as a mechanism of p16 inactivation [100]. In contrast, most T-ALL cases do not appear to have genetic aberrations affecting the expression $p27^{kip1}$, another cdk inhibitor. Nonetheless, homozygous inactivation of $p27^{kip1}$, although probably occurring very rarely, has also been described in one T-ALL case [101]. Furthermore, heterozygous inactivation of $p27^{kip1}$, resulting in decreased but not absent protein levels, seems to be relatively more common in ALL and appears to be the primary consequence of 12p chromosomal deletions in childhood ALL [101]. Another critical regulator of cell cycle progression and apoptosis, p53, is infrequently mutated in T-ALL at diagnosis [102].

Nonetheless, p53 mutations are associated with relapse and poor clinical outcome [100,103] in T-ALL, and occur in most leukemic T-cell lines [104].

The Importance of Extracellular Signals for T-ALL Expansion

As described above, a significant amount of data supports the notion that members of signal transduction pathways involved in survival or proliferation of T-cell precursors are putative targets for the initiation of the leukemogenic process. This is not surprising, because signal transduction pathways are critical targets of oncogenesis. Mutations leading to cancer development are essentially those that affect repair genes or the transmission of signals that control cell expansion and survival. Molecular lesions that disrupt or overactivate signaling pathways at various levels, from the ligand-receptor interactions to the regulation of nuclear transcription, are potentially oncogenic.

Moreover, cytokine and growth factor production in the local microenvironment, and the differential response to the engagement of the respective receptors on normal *versus* leukemic cells may play a major role in the expansion of the malignant tissue at the expense of normal hematopoietic cells [55]. It is conceivable that a clone in the process of transformation might respond differently than its normal counterpart to the same stimuli, resulting in an additional selective advantage favoring the malignant cells. Normal mitogenic signals could contribute decisively to the abnormal expansion of a malignant population possessing a survival-increasing mutation. Likewise, a clone with a mutation leading to augmented proliferation would have a considerable advantage when receiving microenvironmental or humoral signals mediating survival. External signals would, furthermore, offer time to the leukemic cells for acquiring additional advantageous mutations. A striking example in support of this notion is illustrated by another type of hematopoietic neoplasia. In human follicular B-cell lymphoma, t(14;18) results in deregulated upregulation of Bcl-2 expression, leading to an initially indolent tumor whose expansion is dependent upon availability of normal B-cell growth-promoting signals. Subsequently, the malignant cells undergo additional mutations and a substantially more aggressive tumor emerges [105]. Thus, in a malignant setting the normal growth factors can be utilized by the malignant cell in a way that contributes to the promotion of the disease.

Cytokine Regulation Of Cell Death And Cell cycle Progression

Whatever the exact molecular defects that originate T-ALL, they necessarily converge on their capacity to affect the normal regulation of cell cycle progression and apoptosis. Similarly to any other cancer, T-ALL arises from deregulation of these critical processes involved in the control of cellular expansion, which once subverted allow the malignant clone to expand beyond the limits imposed upon non-transformed cells. To understand in part how T-ALL develops and how external factors might effectively modulate the progression of the disease it is necessary to comprehend how cell cycle and cell death are regulated in the normal cell by both intrinsic mechanisms and external factors such as cytokines.

Programmed Cell Death

Apoptosis or programmed cell death is a multistep, tightly regulated, energy-consuming process associated with loss of mitochondrial integrity, cleavage of intracellular proteins and degradation of chromosomal DNA into fragments of distinct size (DNA laddering), and morphologically characterized by blebbing and shrinking of the nucleus and cytoplasm [106]. During the apoptotic process the integrity of the cellular membrane is maintained but phosphatidylserine, which normally is located in the cytoplasmic portion of the lipid bilayer, is exposed at the cell surface thereby targeting the dying cell for phagocytosis by macrophages. The sequence of events leading to cell death by apoptosis differs significantly from those related to necrosis, in which swelling and bursting of the cell and random degradation of chromosomal DNA precede death as a result of injury. Apoptosis is required for normal cell development and is an essential barrier that a cell undergoing malignant transformation should overcome before acquiring a fully transformed phenotype.

An early and critical event in the apoptotic process is the loss of mitochondrial transmembrane potential, mitochondrial outer membrane permeabilization and subsequent release of cytochrome c, which then binds to and activates the apoptotic

protease-activating factor-1 (Apaf-1). Cytochrome c and Apaf-1 take part in a multiprotein complex, the apoptosome, which also includes the cysteine protease caspase 9. Apaf-1 activates caspase 9, a so-called initiator caspase that triggers a caspase cascade leading to activation of the "executioner" caspases 3 and 7 [107]. Caspase activity further results in cleavage of the inhibitor of caspase-activated deoxyribonuclease (iCAD) and activation of CAD, which mediates DNA degradation during apoptosis [108,109].

Members of Bcl-2 protein family are major evolutionarily-conserved regulators of the apoptotic process (reviewed in [110]). No less than fifteen Bcl-2 family members have been identified in mammalian cells. All of them possess at least one of four conserved family-specific regions, the Bcl-2 homology domains (BH1-BH4). Three subfamilies can be considered, the pro-survival Bcl-2 subfamily (Bcl-2 itself, Bcl-x_L, Bcl-w, Mcl-1, A1, and others), and the pro-apoptotic Bax (including Bak and Bok) and BH3-only (Bad, Bid, Bik, Blk, Bim, amongst others) subfamilies. Their pro- and anti-apoptotic activities are regulated in part by formation of homo- and heterodimers and by titration of each other's functions, implicating that their relative expression is important for cell death regulation. Subcellular localization and activation status of each molecule are also critical. The different Bcl-2 family members are located in the cytosol and intracellular membranes. The balance between pro- and anti-apoptotic members in the outer mitochondrial membrane regulates cytochrome c release from mitochondria [111] (Figure 1). For instance, Bax promotes release of cytochrome c [112], and Bcl-2 prevents apoptosis by blocking cytochrome c release [113] via inhibition of Bax [114]. In addition, Bcl-2 subfamily members may also regulate apoptosis downstream of mitochondrial cytochrome c release by binding to Apaf-1 and inhibiting the ability of the apoptosome to activate caspase 9, an effect antagonized by the pro-death members [115]. Upon stimulation by survival factors such as cytokines, the activity of anti-apoptotic Bcl-2 family members succeeds over the effect of the pro-apoptotic members. Cytokine-mediated cell survival is realized not only by transcriptionally inducing the expression of anti-apoptotic Bcl-2 family genes [116-118], but also through posttranslational modification mechanisms. For example, Bad is inhibited by phosphorylation [119-121], while phosphorylation of certain residues can lead to activation of Bcl-2 [122].

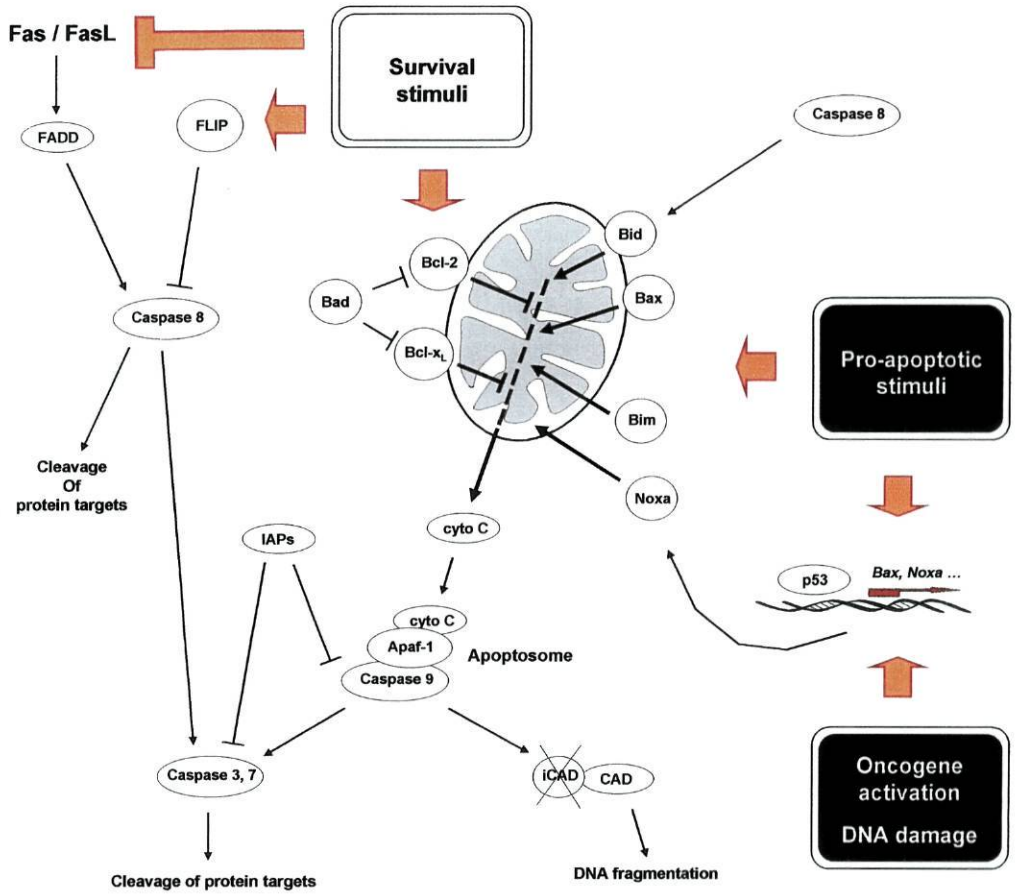

Figure 1. Regulation of apoptosis. Pro-apoptotic Bcl-2 family members promote, whereas anti-apoptotic members prevent, the release of cytochrome c into the cytosol as a consequence of mitochondrial outer membrane permeabilization. Cytochrome c binds to and activates Apaf-1, which then activates caspase 9. Altogether they form a multiprotein complex, the apoptosome. Activation of the "executioner" caspases 3 and 7 is induced by the apoptosome, and results in cleavage of specific protein targets that include the inhibitor of CAD (iCAD). Cleavage of iCAD permits the activation of CAD and leads to DNA degradation. Bcl-x_L and other Bcl-2 subfamily members may also regulate apoptosis by binding to Apaf-1 and sequestering the apoptosome. IAPs directly inhibit caspase activity. In response to oncogene activation, cytokine/growth factor withdrawal or irreparable damage to chromosomal DNA, p53 promotes apoptosis by transcriptionally inducing pro-apoptotic genes, such as Bax and Noxa. The triggering of Fas by FasL leads to activation of caspase 8 after interaction with FADD. Caspase 8 can directly activate caspase 3 and cleave apoptotic substrates. It can also induce the mitochondria/apoptosome cell death pathway (e.g., via activation of Bid). Anti-apoptotic signals can prevent Fas-mediated apoptosis by downregulating the expression of FasL and upregulating FLIP, an inhibitor of caspase 8.

Cytokines and growth factors may also regulate the expression of inhibitor of apoptosis proteins (IAPs), which belong to another evolutionarily-conserved family of

factors that suppress apoptosis by binding to caspases and inhibiting their activities [123]. IAPs are transcriptionally upregulated by nuclear factor (NF)-κB, which is controlled by cytokine-mediated signaling pathways [124-127].

Another important regulator of programmed cell death is the tumor suppressor p53. In response to DNA damage p53 induces cell cycle arrest by transcriptionally upregulating genes such as $p21^{cip1}$ and Gadd45 thereby allowing the cells to repair damaged DNA before progressing in the cycle [128]. In response to oncogene activation, cytokine/growth factor withdrawal or irreparable damage to chromosomal DNA, p53 can induce apoptosis by transcriptional activation of pro-apoptotic genes, such as Bax and Noxa [129,130]. The role of p53 can be positively or negatively modulated by trophic factor withdrawal or stimulation, respectively.

In some cases cytokine withdrawal can prompt apoptosis via surface expression and activation of cell-death receptors, such as Fas/CD95/Apo-1 [131]. Fas is triggered by Fas ligand (FasL/CD95L), which leads to recruitment and activation of caspase 8 (also known as FLICE) via interaction with the adaptor protein FADD. Caspase 8 can directly cleave apoptotic target proteins and directly activate caspase 3, thus mediating apoptosis independently of cytochrome c release and apoptosome activation. In parallel, caspase 8 can also activate the mitochondria/apoptosome cell death pathway [132,133]. As discussed below, cytokines can block Fas-mediated apoptosis by downregulating the expression of FasL and upregulating cFLIP (FLICE inhibitory protein), a molecule that prevents cell death by inhibiting caspase 8 activation (Figure 1).

Cell Cycle Progression

In order to divide, proliferating cells undergo a series of orderly processes called the cell cycle. There are four cell cycle stages. Growth in mass that occurs during Gap 1 (G1) is followed by synthesis/replication of DNA (S-phase), synthesis of proteins required for mitosis (G2 for Gap 2), mitosis (M-phase) and return to G1 or exiting from the cycle into a quiescent state called G0 [134,135]. During cell cycle progression there are certain checkpoints (present in G1, G2 and M) that are essential for a cell to decide between the alternative fates of proliferation, temporary arrest or quiescence, differentiation or apoptosis. The restriction point, the checkpoint that occurs in late G1,

plays a central role in cytokine regulation of proliferation. Before and at this point, cell cycle progression is modulated by extracellular signals such as those elicited by cytokines. Passage through the restriction point commits the cell to DNA replication and cell division, even in the absence of external growth factors [136]. Thus, transition from G1 to S-phase appears to be the most relevant step in cell cycle regulation, since it is affected not only by cell cycle-intrinsic regulatory mechanisms but also by signaling pathways triggered by external factors that control those same mechanisms. Not surprisingly, lesions affecting G1/S regulation are an important cause of cancer, as they permit cells to escape from checkpoint control [137-139]. In fact, cancer has even been defined by some as a disease of the cell cycle [140].

Cell cycle progression is powered by enzymatic complexes formed by regulatory cyclins and catalytic cyclin-dependent kinases (cdks). Higher eukaryotes express at least ten cyclins and nine cdks [141]. Unlike cyclins, which have a short half-life, cdks appear to have a relatively constant expression throughout the cell cycle. Activation of cdks requires binding to cyclins. The association between cyclins and distinct cdks varies in each phase of the cell cycle and results in altered substrate specificity [142]. The various substrates phosphorylated by different cyclin/cdk complexes promote the specific events associated with each phase of the cycle

In the G1-S transition, cdk4 and cdk6 associate with D-type cyclins to promote G1 progression, cyclin E/cdk2 complexes are also involved in G1 progression and commitment to S-phase, and cyclin A/cdk2 initiate and maintain S-phase (Figure 2). Cyclins D, E, and A have been identified as proto-oncogenes and are all rate-limiting for S-phase entry. The ability of cyclin/cdk complexes to induce S-phase depends to a great extent on their capacity to phosphorylate the retinoblastoma protein Rb and possibly other members of the Rb family, namely p107 and p130 [143-145]. Rb blocks the cell cycle by binding to and inhibiting E2F transcription factors. Phosphorylation of Rb by cyclin D/cdk4,6 during G1, cyclin E/cdk2 in late G1, and cyclin A/cdk2 during S-phase results in the release of E2F, allowing the transcription of its target genes, which are necessary for S-phase and cell cycle progression [146,147].

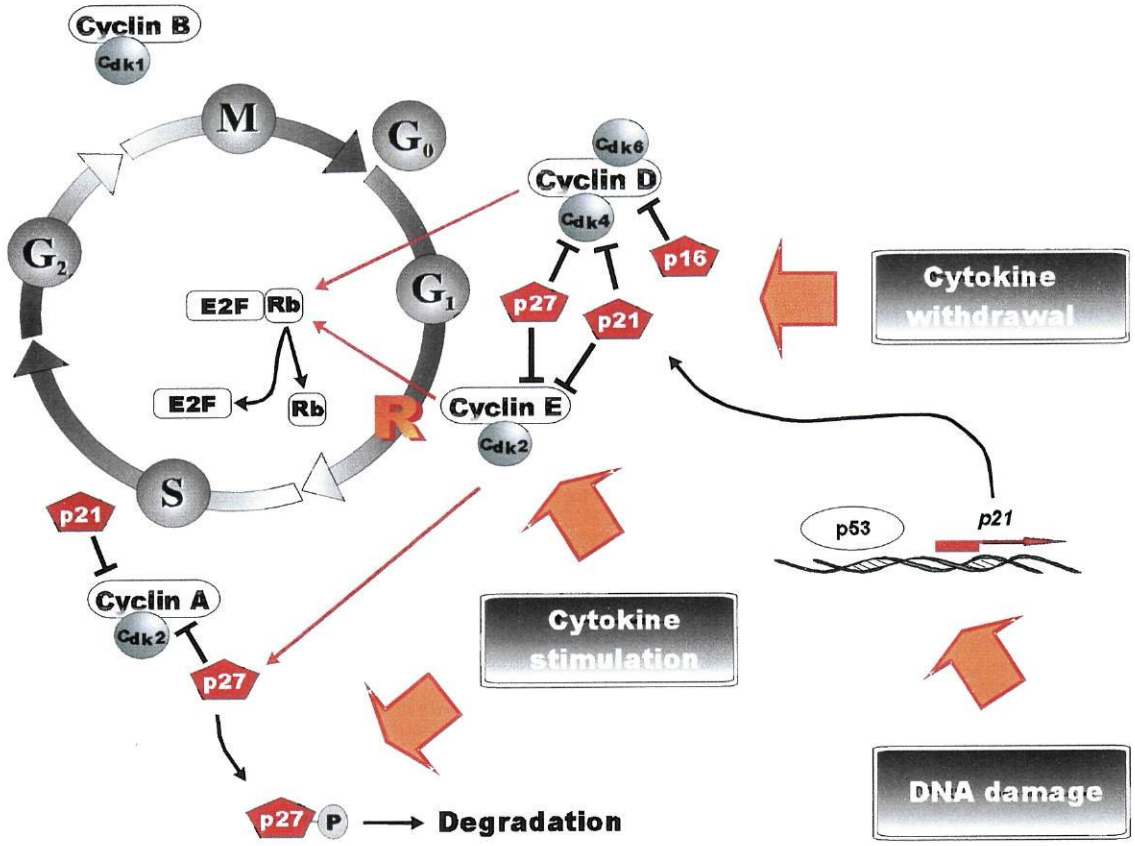

Figure 2. Cell cycle progression: regulation of G1 to S-phase transition. Phosphorylation of Rb by cyclin D/cdk4,6 during G1, cyclin E/cdk2 in late G1, and cyclin A/cdk2 during S-phase results in the release of E2F, allowing the transcription of its target genes, which are necessary to overcome the restriction point (R), enter S-phase and progress in the cell cycle. The activity of cyclin/cdk complexes is prevented by direct binding of cdk inhibitors, which belong to two families: INK4 ($p16^{INK4a}$ is represented here) and the CIP/KIP ($p21^{cip1}$ and $p27^{kip1}$ are represented here). INK4 proteins specifically inhibit cyclin D-dependent kinases cdk4 and cdk6, whereas CIP/KIP proteins inhibit the activities of cyclin D/cdk4,6, cyclin E/cdk2 and cyclin A/cdk2. The levels of $p21^{cip1}$ can be transcriptionally elevated by p53 to promote DNA damage-induced cell cycle arrest, although $p21^{cip1}$ can also be upregulated by mitogenic stimulation, possibly to allow DNA repair of the cycling cells. The levels of $p27^{kip1}$ are strikingly downregulated by cytokine stimulation, as a result of the activation of signaling pathways that mediate its degradation and upregulate the expression and activity of cyclin/cdk complexes.

The activity of cyclin/cdk complexes is inhibited by direct binding of cyclin-dependent kinase inhibitors (CKIs) [148-150]. These cell cycle inhibitors can be divided into two groups based on their structure and cdk targets: the INK4 (inhibitors of cdk4) family, which includes $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ that specifically inhibit cyclin D-dependent kinases cdk4 and cdk6, and the CIP/KIP family, which includes $p21^{cip1}$, $p27^{kip1}$ and $p57^{kip2}$. The ability of INK4 family proteins to arrest cell cycle is dependent on the presence of functional Rb [151], which indicates that by inhibiting cdk4,6 in G1,

INK4 CKIs allow Rb to remain phosphorylated and able to repress S-phase genes [139]. Proteins of the CIP/KIP family have a more broad inhibitory effect, since they affect the activities of cyclin D/cdk4,6, cyclin E/cdk2 and cyclin A/cdk2 (reviewed in [150]). The levels of $p21^{cip1}$ can be transcriptionally elevated by p53 to promote DNA damage-induced cell cycle arrest [128,152,153]. Paradoxally, $p21^{cip1}$ protein expression has been shown to be upregulated by mitogenic stimulation. Since $p21^{cip1}$ also interacts with the proliferating cell nuclear antigen (PCNA), leading to inhibition of DNA elongation by polymerase δ, the increase of $p21^{cip1}$ in proliferating cells has been hypothesized to represent a brief arrest of the cycling cells to allow DNA repair [154-157]. The levels of $p27^{kip1}$ are strikingly downregulated after cytokine stimulation [158] and antisense inhibition of $p27^{kip1}$ expression abrogates the cell cycle arrest induced by growth factor depletion [159], strongly suggesting that $p27^{kip1}$ is a critical sensor that integrates both positive and negative external signals into the cell cycle.

$p27^{kip1}$ is regulated by transcriptional [160,161], translational [162,163] and posttranslational mechanisms, the latter being the most important. In general, the levels of $p27^{kip1}$ mRNA do not appear to fluctuate significantly throughout the cell cycle, in contrast to the protein levels, which decrease as the cycle progresses and are mainly regulated by proteolysis [164]. After the cell passes the restriction point, E2F-mediated expression of S-phase genes such as cyclin E occurs [165]. One of the targets of cyclin E/cdk2 complexes is $p27^{kip1}$ [166]. Phosphorylation of $p27^{kip1}$ on threonine 187 by cyclin E/cdk2 triggers its degradation by the ubiquitin-proteasome pathway [164,167-169]. Other proteins, including Akt/PKB and Erk/MAP kinase, appear to be capable of phosphorylating $p27^{kip1}$ on T187 and other residues, therefore inducing its nuclear export and subsequent degradation [170-172]. Since only when localized in the nucleus can $p27^{kip1}$ bind to active cyclin/cdk complexes and exert its inhibitory effect, sequestration in the cytosol, even without protein degradation, would inactivate $p27^{kip1}$ function. Hence, subcellular localization of $p27^{kip1}$ has been proposed to play a role in its regulation [170,172,173]. Notably, cytoplasmic localization of $p27^{kip1}$ has been reported in various types of malignancies [174,175] and there is some evidence supporting a role for $p27^{kip1}$ cytoplasmic localization in tumorigenesis (reviewed in [176]).

T-cell Development

Since T-ALL cells are defined as the malignant counterparts of T-cell precursors arrested at different stages of differentiation they should reflect discrete stages of normal thymocyte development. The criteria used for the different immunophenotypic classifications of T-ALL cells, including the ones mentioned before (see pages 28-29), are based on this assumption, despite the accepted knowledge that the malignant cells often express aberrant markers indicative of some phenotypic instability and lineage promiscuity [4,31]. Hence, malignant T-cell lines have sometimes been used to study and characterize a particular stage of thymocyte maturation [177]. Conversely, the growing understanding of normal T-cell development has allowed for a more profound discrimination of the stages at which certain T-ALL-associated lesions occur [53].

In the mouse, immature $CD4^-CD8^-$ double negative (DN) thymic lymphoid progenitors are believed to migrate from the bone marrow and enter the thymus in the subcapsular zone. Development within the DN population, which constitutes around 5% of total thymocytes and is mainly $CD3^-$, progresses from the most immature $CD44^+CD25^-$ (pro-T1) stage to $CD44^+CD25^+$(pro-T2), then to $CD44^-CD25^+$ (pro-T3) and finally to the more mature $CD44^-CD25^-$ (pro-T4) stage [178] (Figure 3). Some authors classify $CD44^+CD25^-$ and $CD44^+CD25^+$ as pro-T-cells, and $CD44^-CD25^+$ and $CD44^-CD25^-$ as pre-T-cells [179]. $CD44^-CD25^-$ cells seem to represent the latest developmental stage that still can originate γδ T-cells [180]. Functional rearrangement and expression of TCRβ by the pre-T-cell originates the formation of a pre-TCR complex that includes the pTα chain. Cells with nonproductive TCRβ rearrangements do not express a pre-TCR complex and die. This control point in T-cell development is referred to as β-selection. Signaling through the pre-TCR results in enormous proliferation of the DN thymocytes, which rapidly upregulate CD4 and CD8 to become non-cycling $CD4^+CD8^+$ double positive (DP) thymocytes. This population represents 80-90% of total thymocytes and localizes mainly in the thymic cortex. TCRα gene rearrangement and downregulation of pTα result in TCRαβ surface expression on DP thymocytes.

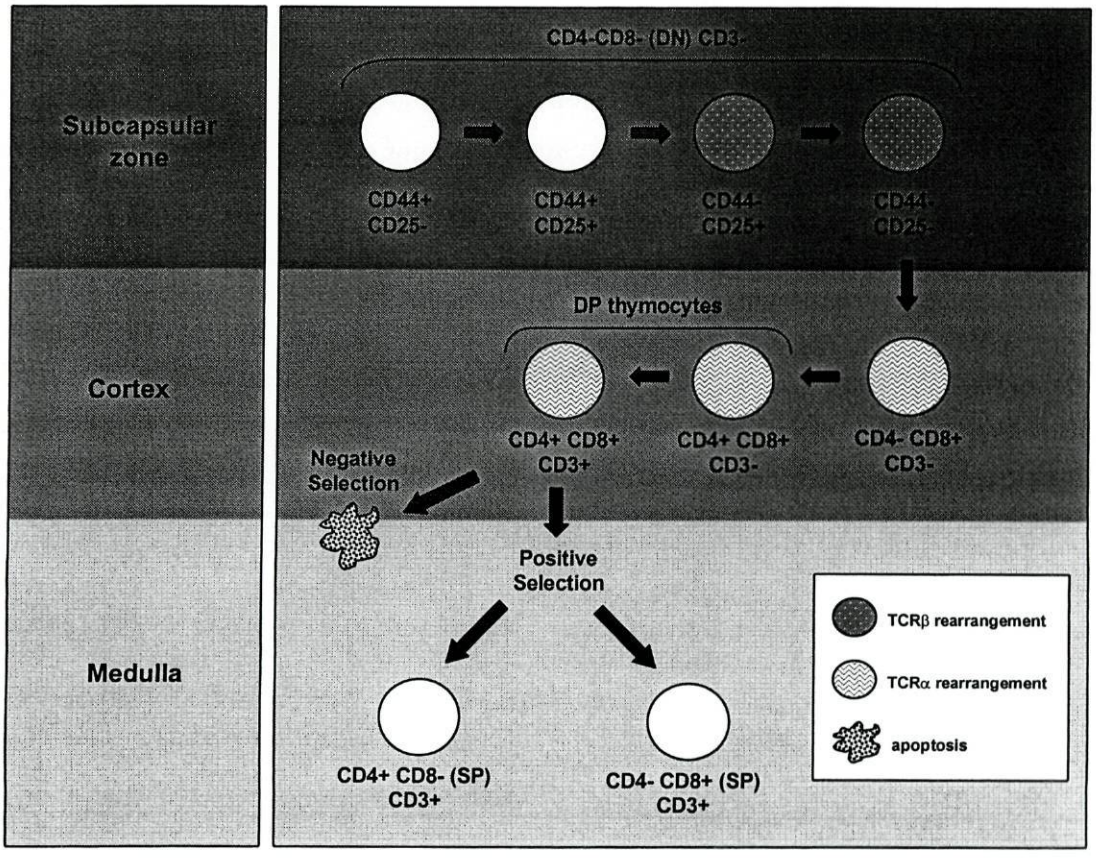

Figure 3. Model of T-cell development in the mouse thymus. CD3- CD4- CD8-T-cell precursors initially develop in the bone marrow and later migrate to the thymus. The most immature thymocytes are present in the subcapsular zone and are CD44+CD25-. Migration towards the thymic cortex and medulla is developmentally regulated and is associated with several processes (pre-TCR expression, β-selection, negative selection, positive selection) that ultimately result in the production of mature CD3+ CD8+ or CD4+ single-positive thymocytes. Detailed explanation in the text.

Maturation into $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ single positive (SP) thymocytes is dependent on the expression of a functional TCRαβ receptor and an appropriate interaction with MHC/peptide complexes expressed at the surface of thymic stromal cells [181]. DP thymocytes undergo massive apoptosis as a result of positive [182] and negative selection [183]. This extremely stringent process of selection proceeds as the thymocytes migrate from the cortex to the medulla and ensures that only the very few DP cells that express a functional TCR complex recognizing self MHC/peptide complexes with a correct avidity will survive and develop further into SP thymocytes. Selected SP

thymocytes proliferate [184] and originate mature SP T-cells that emigrate to the peripheral blood circulation [178]. The entire process is summarized in the model depicted in Figure 3.

Human thymocytes apparently follow a fundamentally similar pattern of development, both regarding immunophenotype, selection processes and the anatomical localization of the several developing subsets (Figure 4). However, some significant differences exist. For example, although human early thymocytes express CD44, the CD25-based sequence used to separate immature DN T-cell precursors is not useful in human T-cell development [185]. Nonetheless, other surface markers, such as CD1, CD2 and CD7, as well as intracellular ones, like cytoplasmic CD3 (cCD3), proved to be quite useful in the human system. Although several and not always coincident developmental schemes have been proposed [32,186-188], some agreement has been achieved as to the main stages of differentiation and the most useful markers to define them.

The earliest intrathymic T-cell precursors express CD34, found in hematopoietic stem cells and early lymphoid precursors, lack CD1 and CD3, and are $CD4^-CD8^-$ DN thymocytes as in the mouse [187]. They further express cCD3 and CD7 and have initially reduced levels of CD2, which quickly increase [186,187,189]. CD5 seems to appear quite early at around the same time as CD2. As DN cells enter the thymic cortex they upregulate CD1 and decrease CD34 expression. Downregulation of CD34 is associated with acquisition of CD4, CD8 and CD3 [187]. CD10, which was initially identified in common ALL, is transiently expressed during the early cortical stages of thymic differentiation. Its expression is maximal at the transition from $CD34^+CD1^+$ to $CD34^-CD1^+$ and is downregulated as CD3 expression increases [53,187]. Human $CD34^+CD1^+$ cells upregulate first CD4 followed by CD8 and then CD3 [187,190], in apparent contrast to the mouse, where CD8 has been proposed to be expressed slightly before CD4 [191]. Hence, in the human thymus the following sequence is observed: $CD3^-CD4^-CD8^-$, $CD3^-CD4^+CD8^-$, $CD3^-CD4^+CD8^+$, $CD3^+CD4^+CD8^+$, in which the initial stages are $CD34^+$ and the latter $CD34^-$ [192,193]. TCRβ gene rearrangements commence in $CD34^+CD1^+$ thymocytes [194] and pTα transcription is maximal in $CD3^-CD4^+CD8^-$ and $CD3^-CD4^+CD8^+$ subpopulations [195]. β-selection occurs at these stages and is accompanied by high proliferative rates [194-196]. Rearrangements of the TCRα locus are initiated at the DP stage [195]. Positive and negative

selection of non-dividing DP thymocytes follows, leading to the generation of small numbers of mature SP T-cells like in the mouse [190].

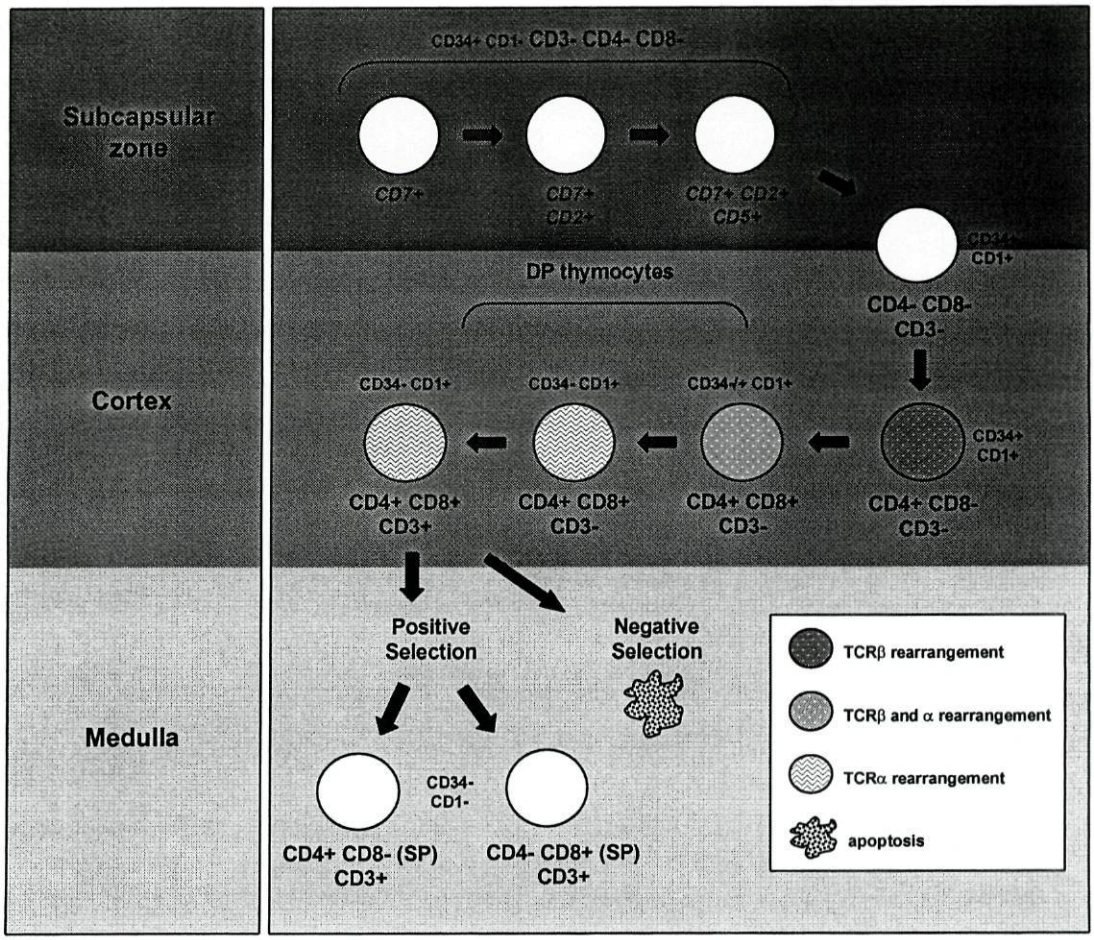

Figure 4. Model of T-cell development in the human thymus. Thymic T-cell development in humans appears to follow the same basic pathway than in mice (pre-TCR expression, β-selection, negative selection, positive selection). However, some surface antigens, such as CD25 and CD44, are not useful in distinguishing human early T-cell precursors. Detailed explanation in the text.

Both murine and human thymocytes in the process of positive selection transiently express CD69, which gets downregulated before migration from the thymus to the periphery [197,198]. Expression of CD38, present throughout T-cell development, is likewise lost just before export to the periphery [187]. Another feature of mature thymocytes is the absence of CD1 surface expression. Downregulation of CD1 marks the transition from the cortex to the medulla and, more importantly, the acquisition of functional

maturity by human thymocytes [199], which are then apt to leave the thymus and expand in the periphery when properly stimulated.

γ_c-Dependent Cytokines And Their Receptors

Interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15, and the newly described IL-21, belong to the IL-2 subfamily of cytokines, whose high-affinity functional receptors share the gamma-common (γ_c) chain [200]. Despite some redundancy, these γ_c-signaling cytokines have essentially distinct and often critical effects in normal T-cell development, survival, proliferation and differentiation. Moreover, they have been directly or indirectly implicated in T-cell leukemogenesis. The role of these cytokines in normal T-cell development and leukemogenesis is reviewed summarily in Chapter 2.

The interleukins of γ_c-signaling group are short-chain (around 130-160 a.a.) type I cytokines, characterized by possessing four α-helical strands with an up-up-down-down topology forming a bundle structure [201,202]. The receptors for these cytokines, with the exception of IL-2Rα and IL-15Rα, are members of the cytokine receptor superfamily, and are also known as type I cytokine receptors or hematopoietin receptors. Members of this superfamily have in common two pairs of conserved cysteine residues that were predicted to be involved in intra-chain disulfide bonding, located near the amino-terminus, and a membrane-proximal WSXWS box (single-letter a.a. code; X is a non-conserved residue), also called WS motif, both in the extracellular domain [203]. There are also sequence similarities amongst type I cytokine receptors in the cytoplasmic domain, in particular the membrane–proximal semi-conserved box1/box 2 region, which is important for signal transduction [204].

The γ_c-signaling cytokines bind to heteromeric receptors lacking intrinsic kinase activity, constituted by γ_c (discussed in page 52) and one cytokine-specific α chain (Figure 5). The receptors for IL-2 and IL-15 share an additional subunit, the IL-2Rβ chain [205]. The structurally similar α chains of IL-2 and IL-15 receptors show no significant homology to other known cytokine receptors, and constitute a unique family

on their own, characterized by the shared Sushi domain [206]. IL-2Rα and IL-15Rα have very short cytoplasmic domains that do not harbor a signal transduction capacity, which is conferred by γ_c and IL-2Rβ, and seem to contribute solely to increasing the binding of the cytokine. The IL-4Rα, IL-7Rα and IL-9Rα are all critical not only for binding but also for signal transduction engagement of the respective cytokine. Their cytoplasmic domains are constituted by several regions, which are differentially involved in recruiting and activating non-receptor protein tyrosine kinases. As a consequence of ligand-binding, activation of these tyrosine kinases occurs, triggering intracellular cascades essential for effects so diverse and crucial as cell cycle progression, gene activation, differentiation or apoptosis.

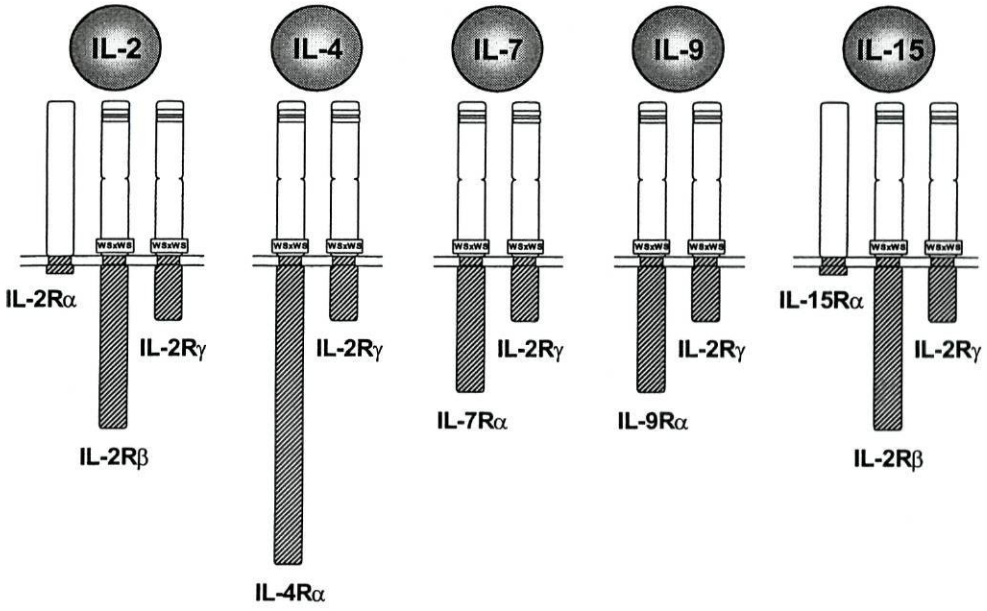

Figure 5. Cytokine receptors that share the γ_c-chain subunit. The four horizontal lines in the membrane-distal portion of the extracellular domain denote the four conserved cysteine residues that are characteristic of the cytokine receptor superfamily, together with the box depicted in the membrane-proximal region representing the WS motif. The two sections in the extracellular portion of all but IL-2Rα and IL-15Rα represent fibronectin-type III-like domains. Extracytoplasmic portions of IL-2Rα and IL-15Rα not represented in detail are subdivided in two and one Sushi domains, respectively, and one Proline/Threonine-rich region. Receptor for IL-21, not depicted here, is constituted by the γ_c-chain and IL-21Rα chain [281]. Adapted from Sugamura et al., 1996 (Ref. [199]).

IL-7 and its Receptor in Normal T-cell biology and Leukemogenesis

Interleukin-7

IL-7 was initially cloned in 1988 as a factor derived from a murine bone marrow stromal cell line [207]. Soon after, the IL-7 human analog was isolated and sequenced. The gene for human IL-7 is located on chromosome 8q12-13 [208], it has a length of approximately 33 kb containing 6 exons and 9 introns and extensive 3' and 5' untranslated regions, with an open-reading frame of 534 bp (177 a.a. including a 25-a.a. secretory signal) [209]. The murine IL-7 gene has around 80% homology to the human gene in the coding regions but lacks the sequence corresponding to the human exon 5, thus resulting that the human protein is 17 amino acids longer, with a predicted molecular weight of 17.4 kD. However, glycosylation results in a 25 kD active form. Murine and human IL-7 share 65% amino acid sequence identity, and exhibit cross-species reactivity. Human IL-7 is clearly active on mouse cells [210]. Although it has been suggested that murine IL-7 does not stimulate human cells [211] because it fails to induce proliferation of human bone marrow cells *in vitro*, there is indirect [212] and direct [213] evidence that murine IL-7 can stimulate human T lymphocytes.

IL-7 is produced by stromal cells of the thymus and bone marrow [214,215]. The thymus has been reported as the anatomical site of highest IL-7 production [214]. Both cortical and medullary MHC class II- expressing thymic epithelial cells (TEC) have been shown to synthesize IL-7 [214,216,217]. Other cell types produce IL-7, including the intestinal epithelium [218,219], keratinocytes [220], follicular dendritic cells and vascular endothelial cells [221]. Our group has recently observed that BM endothelial cells and mesenchymal stem cells also express IL-7 (Yunes and Cardoso, manuscript in preparation).

The identification of IL-7 as a growth factor for B-cell progenitors [222] was followed by the realization that IL-7 could mediate proliferation of both murine and human thymocytes [223,224]. Since then, extensive studies demonstrated that the effects of IL-7 upon developing thymocytes are multiple and non-redundant. Studies with genetically manipulated mice and humans with severe combined immuno-deficiencies (SCID) revealed the fundamental role of IL-7 in T-cell development. Mice deficient for IL-7, IL-7R and γ_c have comparable phenotypes, with severe lymphopenia as a result of

impaired T- and B-cell development and complete absence of γδ T-cells (see below), suggesting that IL-7 has differential effects on TCRαβ and TCRγδ developing T-cell precursors. IL-7R -/- mice have a relatively more severe phenotype than IL-7 -/- mice, which has been suggested to be due to the effect of thymic stromal-derived lymphopoietin (TSLP), a cytokine whose receptor includes the IL-7Rα chain and a specific TSLPR chain [202]. Mice treated with anti-IL-7 antibody for 12 weeks showed a striking reduction in thymic cellularity and an arrest in differentiation at the DN $CD44^+CD25^+$ stage, similar to IL-7 -/- mice [225]. In accordance, mice treated with anti-IL-7R antibody for a shorter period showed a significant decrease in thymocyte numbers [226]. In humans, defective IL-7R expression results in $T^-B^+NK^+$ SCID [227,228]. Furthermore, X-linked SCID (X-SCID), which is characterized by a profound or complete T-cell deficiency and normal or slightly increased B-cell numbers, results from mutations of the γ_c [229]. Consequent lack of IL-7 signaling has been implicated in the pathogenesis of the disease [230,231]. The non-redundant, crucial role of IL-7 is obvious in both human T-cell and murine T- and B-cell development. In contrast, IL-7 does not appear to play a fundamental role in human B-cell development.

Early work using recombinant IL-7 demonstrated that IL-7 is capable of inducing proliferation of human [224,232-234] and mouse [223,235,236] thymocytes in a direct way [223,232]. Several thymic subpopulations have been shown to proliferate in response to IL-7 stimulation including CD34+CD7+ early T-cell precursors [233], $CD4^-CD8^-$ DN thymocytes [223,232,235,236] and $CD4^+/CD8^+$ SP mature thymocytes [184,235], but not $CD4^+CD8^+$ DP thymocytes [235]. The effect of IL-7 on the latter subpopulation is subject of some controversy since human $CD4^+CD8^+$ DP thymocytes were suggested to be capable of proliferating when stimulated by IL-7 and subsequently differentiating mainly towards a $CD4^+CD8^-$ phenotype [224]. However, the data presented to support this assumption do not distinguish between IL-7-induced cell cycle progression and prevention of apoptosis. Since DP thymocytes are essentially non-dividing cells, it is possible that the effect of IL-7 on human $CD4^+CD8^+$ cells is associated with maintenance of this population rather than expansion. In this regard it should be noted that DP cells, in contrast to DN and SP, were originally reported to lack expression of IL-7R [226]. However, detailed analysis showed that most DP thymocytes undergoing positive selection express IL-7R.

Appropriate signals from the IL-7R and TCR, assure the survival of DP thymocytes at least in part through upregulation of Bcl-2 [237,238]. Recent work further supported the notion that IL-7 plays a fundamental role during positive selection [239].

While the effect of IL-7 on thymocyte cell cycle progression remains unsettled [240], and IL-7 *in vivo* proliferative effect might be achieved due to combination with other factors such as Steel factor (SF) [232,241], it is evident that IL-7 is a survival factor essential for normal T-cell development. Initial studies demonstrated that IL-7 enhances viability of thymocytes in fetal thymic organ cultures (FTOC) [236,242]. Importantly, expression of Bcl-2 during T-cell ontogeny coincides with the expression of IL-7R [211]. DN and SP thymocytes, which have high IL-7R expression, present high Bcl-2 levels, whereas DP thymocytes express low levels of IL-7R and Bcl-2. Furthermore, absence of IL-7 signaling results in reduction of endogenous Bcl-2 levels both in thymocytes and in mature T-cells [237], indicating that IL-7 functions as an antiapoptotic factor by regulating Bcl-2 expression. Mice deficient in IL-7R are highly lymphopenic due to a defect in cell expansion at an early stage of differentiation and the few mature T-cells that develop are functionally impaired [243]. Strikingly, these defects are rescued by overexpression of Bcl-2 [117,244]. IL-7 -/- mice exhibit a similar defect in lymphopoiesis, with reduced thymic cellularity, dramatic loss of Bcl-2 protein expression and increased proportion of $CD44^{+}CD25^{+}$ DN thymocytes in G0/G1 stage of the cell cycle [245,246]. Culture of immature thymocytes from IL-7 deficient mice with IL-7 caused upregulation of Bcl-2 protein and cell survival [246]. Thus, IL-7 mediated signals are linked to prevention of apoptosis and cell cycle progression at a transition point in early T-cell development, prior to antigen receptor rearrangement (see Figure 3 for developmental stages). The survival effect mediated by IL-7 during thymocyte maturation seems to involve also regulation of the pro-apoptotic Bcl-2 family member Bax [240,247]. In view of IL-7 positive role in T-cell viability and proliferation it is not surprising that IL-7 has also been implicated in leukemogenesis. Indeed, IL-7 transgenic mice have been shown to develop T and B lymphoid malignancies thus suggesting that IL-7 can act as a T-cell oncogene *in vivo* [248].

IL-7 appears to be directly involved in the induction of TCRγ gene rearrangement by controlling the accessibility of the TCRγ locus through STAT5 and histone acetylation

and demethylation [249-253]. Although several studies proposed that IL-7 might control other TCR loci [216,254,255] (reviewed in [256]) it has been difficult to unequivocally show whether IL-7 actually controls the process of gene rearrangement or simply enhances the viability of thymocytes that are undergoing TCR rearrangements because these effects occur concomitantly.

The evident relevance of IL-7 for TCRγ gene rearrangement, together with the effect on viability of TCRγδ lymphocytes [235], might explain why IL-7 is absolutely required for the development of γδ T-cells. In fact, these lymphocytes are completely deleted in IL-7R -/- [257,258] and IL-7-/- mice [259]. Moreover, IL-7 plays an essential role in the generation and maintenance not only of thymus-derived but also of extrathymic-generated γδ T-cells [219,260].

One interesting and unexplored role that has been suggested for IL-7 during thymopoiesis is the involvement in microenvironmental changes induced by thymocytes. In fact, IL-7 *in vitro* stimulation of human thymocyte cell lines has been shown to consistently upregulate the angiogenesis-related gene vascular endothelium growth factor (VEGF) [261]. If this role proves to be tangible *in vivo,* it will have obvious implications for normal thymic development and the putative involvement of IL-7 in leukemogenesis.

Age-associated thymic atrophy is thought to result from a failure of the thymic microenvironment to support thymopoiesis and it was hypothesized that a decline in IL-7 synthesis may limit thymocyte development. Treatment of aged mice with IL-7 induced significant increases in $CD3^-CD4^-CD8^-$ thymocytes [262]. However, there are conflicting reports observing either no decline [263] or a decrease with age [264] in IL-7 mRNA expression. Thus, IL-7 may enhance thymic function during aging, although it seems unlikely that decreased levels of IL-7 are the sole cause for the age-resulting decline in thymus performance [178].

IL-7 is also a potent modulator of mature T-cell function. Although on its own IL-7 is not mitogenic for mature peripheral blood T-cells, IL-7 is a costimulator for T-cell activation by enhancing proliferation and cytokine production [265-267]. Similar to its effect on thymocytes, IL-7 is an anti-apoptotic factor for mature T-cells [268-270]. Finally, IL-7 mediates the homeostatic proliferation and survival of naïve and memory T-cells [271-274]

and directly enhances the generation and lytic activity of cytotoxic T lymphocytes and γδ T-cells [178,275].

The IL-7 Receptor Complex

As mentioned above, the receptor for IL-7 consists of at least two subunits, IL-7Rα and γ_c [276,277]. The possibility of existing a third, as yet unidentified, subunit derives from reconstitution studies with human IL-7Rα and γ_c that revealed three affinity classes of IL-7R, a high-affinity complex constituted by the two different subunits, an intermediate-affinity complex consisting of binding to IL-7Rα alone, and a low-affinity uncharacterized complex [278,279].

The γ_c subunit was initially cloned as the γ chain from the IL-2R complex [280], and subsequently proven to be a functional component of the receptors for IL-4 [281], IL-7 [277], IL-9 [282], IL-15 [205], and more recently IL-21 [283]. The mature form of γ_c consists of 347 a.a. residues, with an extracellular region 232 a.a.-long, a single transmembrane domain with 29 a.a., and an 86 a.a.-long cytoplasmic region containing two Src homology region 2 (SH2) subdomains, which however do not constitute a fully functional SH2 domain [200]. The γ_c is expressed in a constitutive manner on virtually all cells of hematopoietic origin. It functions to enhance the cytokine binding, presumably by direct contact with the ligand [284], and to transduce signals trough Jak3, a non-receptor protein tyrosine kinase specifically associated with the membrane-distal region of γ_c cytoplasmic tail [285]. In fact, humans with Jak3 mutations present an autosomal SCID syndrome similar to X-SCID [286,287], while Jak3- and γ_c-deficient mice share a virtually identical phenotype [288-290].

IL-7Rα, which directly binds IL-7, is constituted by an extracytoplasmic region with 220 a.a. residues, a 25 a.a. transmembrane region, and a 195 a.a. cytoplasmic tail that plays a critical role in intracellular signaling [291]. The cytoplasmic portion of the IL-7Rα contains three subdomains: a membrane-proximal acidic region, a serine-rich domain and a membrane-distal tyrosine-containing region, all of which have been implicated in associating with signal transduction molecules [292]. Similar to the other hematopoietin receptors, the cytoplasmic portion of IL-7Rα has a box 1 motif but lacks a box 2 [204]. Heterodimerization of IL-7Rα and γ_c is necessary and sufficient for activation

of intracellular signaling events [230,293], whereas the homodimerization of IL-7Rα appears to be insufficient for signal transduction [293].

IL-7-triggered Intracellular Signaling Pathways

Current knowledge suggests that IL-7 binds to IL-7Rα and possibly γ_c, leading to heterodimerization of the two IL-7R complex subunits. The γ_c-associated Jak3 is consequently activated by a mechanism which is still elusive and that might be the result of autophosphorylation related to receptor dimerization and/or conformational changes. Activated Jak3 phosphorylates Jak1, another tyrosine kinase of the same family as Jak3 that is associated with the box 1 region of IL-7Rα [200]. The proximity between IL-7Rα and γ_c permits Jak3 to further phosphorylate tyrosine residues in the cytoplasmic region of IL-7Rα leading to recruitment of signal transducers and activators of transcription (STAT) proteins as well as other intracellular signaling molecules (Figure 6) that bind to the receptor phosphorylated tyrosine residues through their SH2 domains. Although γ_c is critically necessary for IL-7 signaling, this requirement appears to be mainly due to the need for Jak3 to phosphorylate IL-7Rα subunit and IL-7Rα-associated proteins [230]. In other words, γ_c serves primarily to initiate signal transduction by the IL-7R complex, while IL-7Rα determines specific signaling events through its association with cytoplasmic signaling molecules. Nonetheless, γ_c is physiologically indispensable for IL-7 signaling in hematopoietic cells, and the γ_c-associated Jak3 mediates the activation of downstream targets that have been shown to associate both with IL-7Rα and directly with Jak3 [294]. This suggests that the IL-7-specific signals are triggered mostly by the IL-7Rα but also by the unique interaction between IL-7Rα, γ_c and the downstream molecules recruited to the cytoplasmic portions of the heterodimeric receptor.

Early work demonstrated that IL-7 induces tyrosine phosphorylation of several intracellular proteins in normal and leukemic T-cells and thymocytes [224,234,295]. Although a number of these proteins have been identified, their particular role in IL-7-mediated signaling is in some cases still very ill defined.

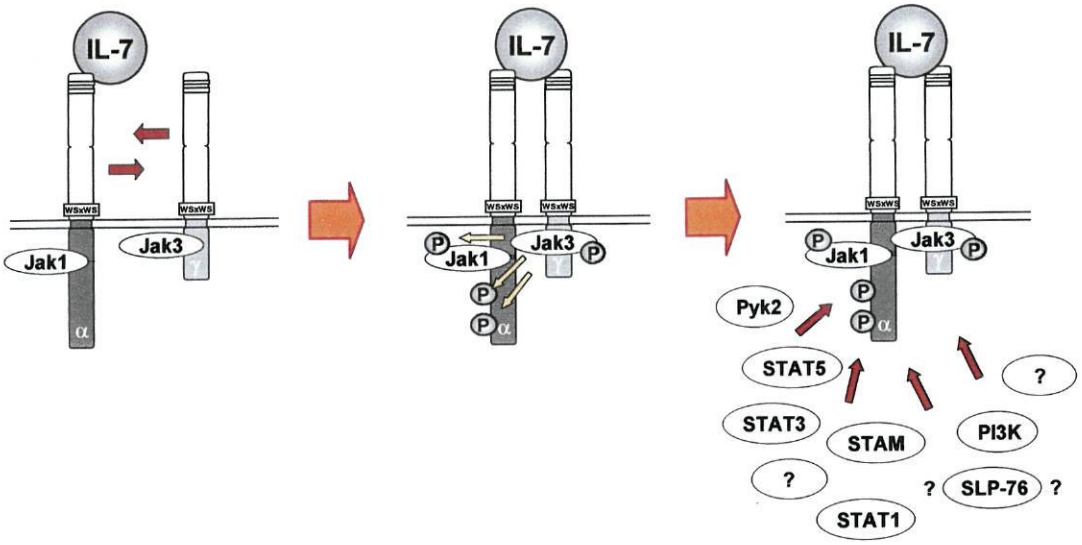

Figure 6. Initial steps of IL-7-mediated signaling. IL-7 binds to IL-7Rα thereby recruiting γ_c, and leading to heterodimerization of the two IL-7R subunits. γ_c contributes to increase the affinity of the IL-7R towards IL-7, possibly via direct binding of IL-7. The γ_c-associated Jak3 is phosphorylated and activated as a consequence of IL-7 binding. Activated Jak3 phosphorylates Jak1. The proximity between IL-7Rα and γ_c allows Jak3 to phosphorylate tyrosine residues in the cytoplasmic region of IL-7Rα, leading to recruitment of different downstream targets that bind to the receptor phosphorylated tyrosine residues and switch on downstream signaling cascades.

Some of the Pieces of an Incomplete Puzzle

The Src family members $p56^{lck}$ and $p59^{fyn}$ protein tyrosine kinases appear to be constitutively associated with IL-7Rα in primary human thymocytes and mature T-cells. Stimulation by IL-7 leads to increased activation of both tyrosine kinases, according to some authors [296] or solely $p56^{lck}$ but not $p59^{fyn}$ according to others [294]. Nonetheless, $p59^{fyn}$ does not seem to mediate the most critical effects of IL-7 on T-cell development, since $p59^{fyn}$ -/- mice do not show an overt phenotype and do not present the T-cell developmental impairment associated with IL-7/IL-7R/γ_c deficiencies [297]. $p56^{lck}$ -/- mice have impaired DP thymocyte development [298], which is better explained by $p56^{lck}$ critical role in TCR-mediated signaling [299], necessary for T-cell positive selection, than by absence of IL-7-mediated signals. Moreover, contrary to IL-7- or IL-7R -/- mice, p56 deficiency does not abrogate γδ T-cell development although affecting the expansion and

repertoire of γδ T-cells [300]. Finally, IL-7-induced $p56^{lck}$ and $p59^{fyn}$ activity does not correlate with proliferation in mature T-cells [296]. Therefore, the role of $p56^{lck}$ and $p59^{fyn}$ in IL-7-mediated signal transduction, and consequently T-cell proliferation, survival and development remains to be clearly defined.

Pyk2, a protein tyrosine kinase related to focal adhesion kinase, has been shown to be rapidly tyrosine phosphorylated and activated in response to IL-7 in murine thymocytes. Pyk2 was found to be physically associated with Jak1 and IL-7Rα, and to increase its association with IL-7Rα after IL-7 stimulation. Furthermore, Pyk2 appears to be involved in mediating cell survival [301], although Pyk2 downstream targets associated with this effect remain unknown.

Signal transducing adaptor molecule (STAM) was first shown to be tyrosine phosphorylated rapidly in response to IL-7, and suggested to act as an adaptor molecule involved in signal transduction from cytokine receptors [302]. STAM was further implicated in cell growth signaling and c-Myc induction immediately downstream of Jaks [303]. Recently, STAM2 a new member of the same family was identified and demonstrated to have a similar role to STAM in cytokine signal transduction [304,305]. However, the pathway connecting IL-7 and STAMs to c-Myc expression and cell proliferation is still unresolved.

Other molecules suggested to play a putative role in IL-7 signaling include the adaptor protein SLP-76 [211] and RhoGTPase [306], but no direct evidence was ever shown regarding their involvement in IL-7-mediated cellular effects.

Jak/STAT Pathway

The pathway involving Jak (Janus kinase) protein tyrosine kinases and STAT transcription factors is typically activated in response to growth factor stimulation and plays a critical role in cytokine signaling. Although cytokines were shown to activate other pathways, none has been so clearly and specifically associated with cytokine-receptor signaling as the Jak/STAT pathway. The discovery of Jak and STAT homologues in *Drosophila* and *Dictyostelium* implies an ancient evolutionary origin for this phylogenetically conserved pathway [307,308] that denotes its critical biological

function. Strikingly, studies in *Drosophila* have shown that Jak mutations, known as *Tum-l* (tumorous lethal), result in developmental defects and leukemia [309,310].

Jak Tyrosine Kinases

In mammals, four Jak family members have been identified so far: Jak1, Jak2, Jak3 and Tyk2 [311]. Jak3 is expressed primarily in hematopoietic cells, while the other three are more widely expressed. Jaks are relatively large (120-140 kD) proteins with seven conserved regions within the family (JH1-JH7) (reviewed in [312]). JH1 and JH2 are tandem kinase and kinase-like domains, respectively. The carboxyl-terminal JH1 is a fully functional catalytic domain. JH2 is a pseudokinase domain whose precise function has not been determined yet [313], although several lines of evidence suggest that it plays a role in regulating Jak catalytic function [201] and/or serves as a docking site for STATs and other signaling molecules [314]. The term Janus kinase substituted the rather blunt initial meaning of Jak, "just another kinase", and refers to the fact that, similar to the Roman mythological god Janus, Jaks also have "two faces" consisting of the two kinase-like domains. Jaks have no transmembrane or membrane-binding domains, and lack SH2 or SH3 domains. However, the amino-terminal domains (JH5-JH7) have the capacity for binding to the cytoplasmic region of the cytokine receptor [201,312]. Interestingly, Jaks have an SH2-like segment in their JH4 domain whose ability to bind phosphotyrosine residues or have a clear impact on downstream signaling has not been demonstrated until now.

STAT Transcription Factors

Seven STAT proteins have been characterized in mammalian cells STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 [201]. STATs share several conserved regions that include an amino-terminal domain, a coiled-coil domain, a DNA binding domain, a linker domain, and an SH2 domain. Carboxyl terminal to the SH2 domain there is a conserved tyrosine residue and a transcriptional activation domain (TAD) whose sequence is not conserved (reviewed in [308]). With the exception of STAT2 and STAT6, STATs possess also a serine residue close to the carboxyl terminus. Phosphorylation of this residue, which probably occurs in the cytoplasm, is important for full activation of STAT transcriptional activity [307]. STAT proteins interact physically

and/or functionally with a number of other nuclear proteins and transcription factors, including p300/CBP, Nmi, SMAD1 and 4, NF-κB, and AP-1, which appear to either facilitate and stabilize DNA binding or increase STAT transcriptional activity without affecting the association with DNA (reviewed in [315]).

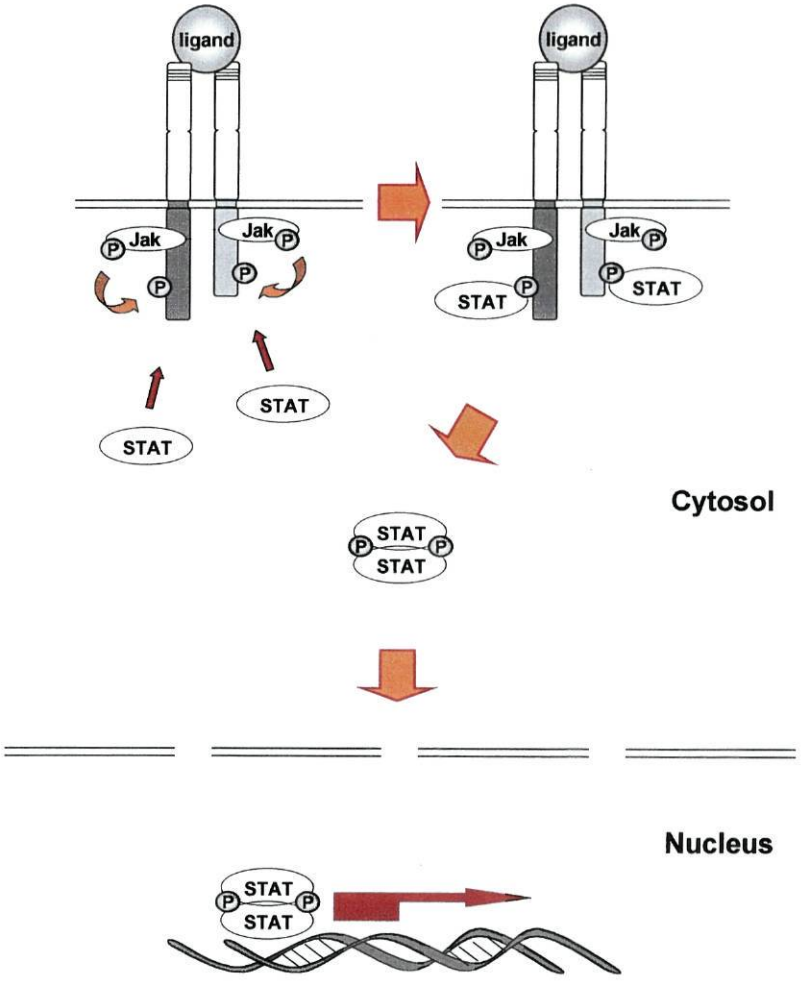

Figure 7. The Jak/STAT pathway. Ligand binding to the receptor induces activation of Jak tyrosine kinases, which in turn phosphorylate the receptor chains at tyrosine residues, thereby providing docking sites for the SH2 domains of STAT monomers. The STATs are subsequently tyrosine phosphorylated by the Jaks, inducing STAT dissociation from the receptor and dimerization. The STAT dimers are then able to enter the nucleus, where they bind DNA consensus sequences and mediate trancription of target genes.

Activation of STATs by Jaks

As described above for IL-7, the binding of a cytokine to its receptor rapidly induces activation of Jak proteins and immediate tyrosine phosphorylation of the receptor by the activated Jaks. Phosphotyrosine-based motifs in the cytoplasmic tail of the receptor provide docking sites for STATs that are constitutively located in the cytoplasm. Receptor-bound STATs are tyrosine phosphorylated by the Jaks. Phosphorylation of STATs originates their release from the receptor and allows homo- or heterodimerization to occur. The dimeric form is then able to translocate into the nucleus, where it binds to DNA consensus sequences and activates the expression of target genes which are involved in a variety of cellular functions, including proliferation, survival and differentiation as well as specialized cellular events [315,316] (Figure 7). Target genes regulated by STATs include, amongst many others, Bcl-2 family members, cyclin D1, $p21^{cip1}$, IL-2Rα, and c-Myc [201,316,317].

IL-7 and Jak/STAT Pathway

IL-7 has been shown to induce the rapid phosphorylation and activation of Jak1 and Jak3 in murine and human T-cells [294,318,319], independently of Src family ($p56^{lck}$ or $p59^{fyn}$) kinase activity [294]. STAT5 directly associates with phosphotyrosines in the tyrosine-containing membrane-distal cytoplasmic region of IL-7Rα [292]. IL-7 stimulation results in STAT5a and STAT5b tyrosine phosphorylation, homo- or heterodimerization, rapid translocation into the nucleus and increased DNA binding activity [213,319,320]. To a lesser extent, IL-7 has been demonstrated to activate STAT1 and STAT3 [213,320,321].

The biological consequences of IL-7-mediated activation of Jak/STAT signaling pathway in thymocytes and mature T-cells remain somewhat elusive. Jak1 -/- mice present severely impaired T-cell development, with reduced size thymi and highly diminished thymocyte numbers, which was shown to be the consequence of IL-7R dysfunction caused by the absence of Jak1 [322]. The striking similarity between the phenotypes of Jak3-, IL-7- and IL-7R-deficient mice suggest a direct link between IL-7 and Jak3 signaling during T-cell development. Moreover, activation of Jak1 and Jak3 was suggested to play a key role in IL-7-mediated proliferation of T-cells [319]. However, STATs are not the exclusive downstream targets of Jaks. Other molecules, such as PI3K,

could account for Jak1 and Jak3 effects on T-cell development and proliferation. In fact, one report demonstrated that mice lacking both STAT5a and STAT5b do not appear to have impaired thymic development [323], and although another study observed that STAT5b -/- mice have somewhat reduced thymic cellularity [324], the phenotype is not comparable to that of Jak3-, IL-7- or IL-7R-deficient mice. Since STAT5 is the most important IL-7-activated STAT, this suggests that the Jak/STAT pathway is not fundamental, by itself, for IL-7-mediated T-cell development, although it may still have a non-critical contribution perhaps in concatenation with other pathways. Notwithstanding, STAT5 has been implicated in IL-7-mediated TCRγ gene rearrangement and consequently γδ T-cell development [251,253]. Unfortunately, the studies dealing with STAT5a/b deficient mice have only assessed TCRαβ T-cells, and thus one cannot conclude on the actual relevance of STAT5 for IL-7-dependent γδ T-cell development. Importantly, it is possible that STAT5 requirement for human T-cell development is different from mice. In this regard, it has been elegantly demonstrated that IL-7-mediated activation of STAT5 induces differentiation of human thymocyte precursors in FTOC [325].

Despite showing apparently normal thymic development, mature T-cells from STAT5a/b -/- mice have impaired mitogenic responses to TCR and IL-2 stimulation [326] Moreover, STAT5 pathway has been shown to act synergistically with other pathways to maximize IL-2-mediated cell growth [327]. The fact that the IL-2R promotes lymphocyte proliferation and induction of genes involved in proliferation (c-Myc) and survival (Bcl-2 and Bcl-x) through the transcriptional activation domain of STAT5 [317] raises the possibility that IL-7-mediated upregulation of c-Myc and Bcl-2 might be achieved by the same mechanism. Interestingly, studies with STAT3-deficient T-cells have implicated STAT3 in prevention of apoptosis through a Bcl-2-independent mechanism [328]. Thus, Jak/STAT pathway may contribute to T lymphocyte increased survival and proliferation induced by IL-7.

MAPK Pathways

Mitogen-activated protein (MAP) kinases take part in different signaling modules that are activated by a wide range of extracellular stimuli including cell-cell contact, growth factors, cytokines, hormones and environmental stresses, thereby contributing to

the integration, amplification and control of signals affecting cell proliferation, differentiation and apoptosis as well as tissue-specific responses such as inflammatory or acute hormonal responses [329,330]. MAP kinase signaling modules are organized as signaling cascades in which MAP kinases (MAPK) are activated by dual-specificity (serine/threonine and tyrosine) MAPK kinases (MAPKK), which in turn are activated by multiple MAPKK kinases (MAPKKK) (Figure 8). Three major MAPK pathways have been identified. Namely, the extracellular-signal regulated kinase (Erk) pathway, which is predominantly activated by mitogenic stimulation, and the c-Jun N-terminal kinase (JNK) / stress-activated protein kinase (SAPK) and $p38^{MAPK}$ pathways that are stimulated by environmental and genotoxic stress [329].

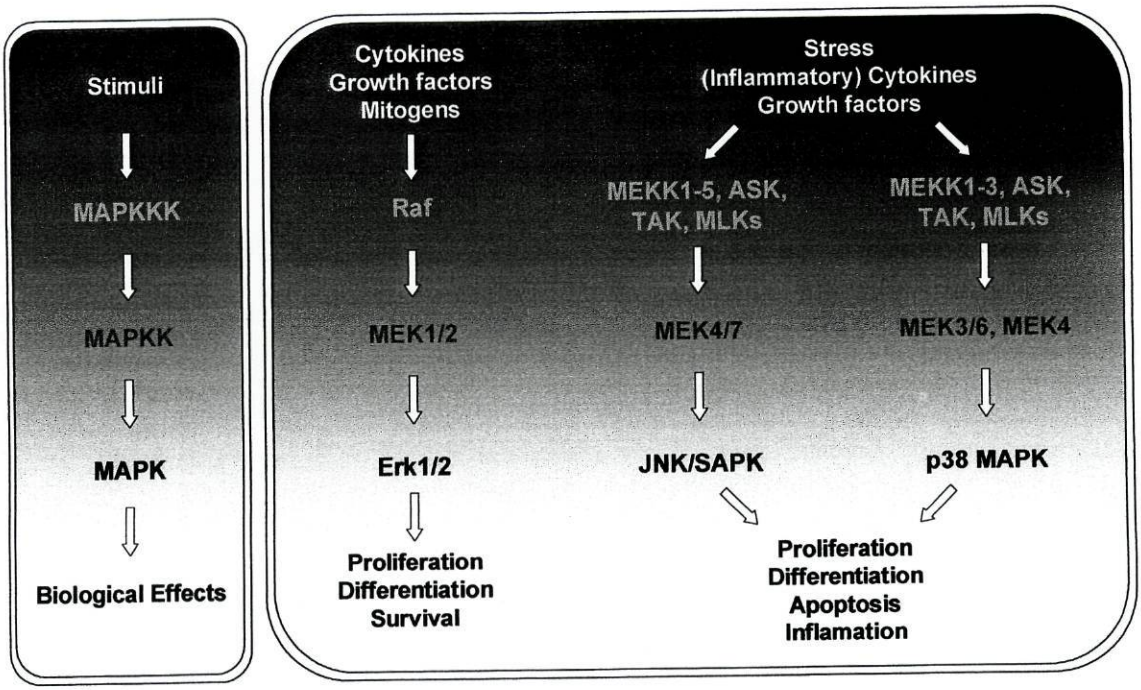

Figure 8. The MAP kinase signaling modules. The three major MAPK pathways, Erk, JNK/SAPK and $p38^{MAPK}$ signaling modules, are organized as cascades in which a MAPKK kinase (MAPKKK) activates a MAPK kinase (MAPKK), which in turn activates a MAP kinase (MAPK) that phosphorylates downstream targets (transcription factors, other kinases), which then contribute to a defined biological response. The Erk pathway is predominantly activated by mitogenic stimulation, whereas JNK/SAPK and $p38^{MAPK}$ pathways are mostly stimulated by environmental and genotoxic stress. Increasing evidence suggests the existence of significant cross-talks between the different MAPK signaling pathways.

p44/p42 MAPK (Erk1/2) Pathway

Raf/MEK/Erk cascade constitutes the prototypical MAPK signaling pathway that is activated in response to non-inflammatory cytokines. Tyrosine phosphorylation of the cytokine receptor cytoplasmic tail following ligand binding provides docking sites for recruitment of the adapter protein Shc. Once associated with the receptor Shc is tyrosine phosphorylated and, in turn, recruits Grb-2. This adapter protein contains a single SH2 domain flanked by two SH3 domains and can be, in many cases, directly recruited to the receptor without Shc intervention. Grb-2 associates, via the SH3 domain, with the Ras guanine nucleotide exchange factor SOS (Son of Sevenless) and the Grb-2/SOS complex is targeted to the plasma membrane where SOS catalyzes the exchange of GDP for GTP on Ras. Replacement of GDP with GTP activates Ras, a 21kD membrane-bound small GTPase which is a central player in signal transduction and is mutated in about one third of all human cancers [331]. RasGTP has several downstream targets, including c-Raf, a serine/threonine MAPKKK that is recruited to the membrane and subsequently phosphorylated and activated. Some other protein kinases, including Protein Kinase C (PKC) [332] and Src [333], phosphorylate Raf, possibly in a Ras-independent manner. Activated Raf phosphorylates MEK, the only known *in vivo* target of Raf [334]. Two related genes encode MEK1 and MEK2 [335]. MEK1/2 are dual-specificity MAPKKs that activate $p44^{MAPK}$/Erk1 and $p42^{MAPK}$/Erk2 by phosphorylating tyrosine and threonine residues on a TEY (threonine-glutamate-tyrosine) motif of their activation loop. MEK1/2 do not phosphorylate either JNK/SAPK or $p38^{MAPK}$, the two main stress-activated MAP kinases. Upon activation, Erk1/2 phosphorylate targets in the cytoplasm, such as $p90^{RSK}$ [336], Mnk [337] and Msk [338] protein kinases, and subsequently translocate to the nucleus, where they mediate the activation of transcription factors that include Elk-1, c-Myc, c-Jun, c-Fos and C/EBPβ (reviewed in [339]), thereby controlling immediate/early gene expression. The p44/p42 MAPK pathway is summarized in Figure 9.

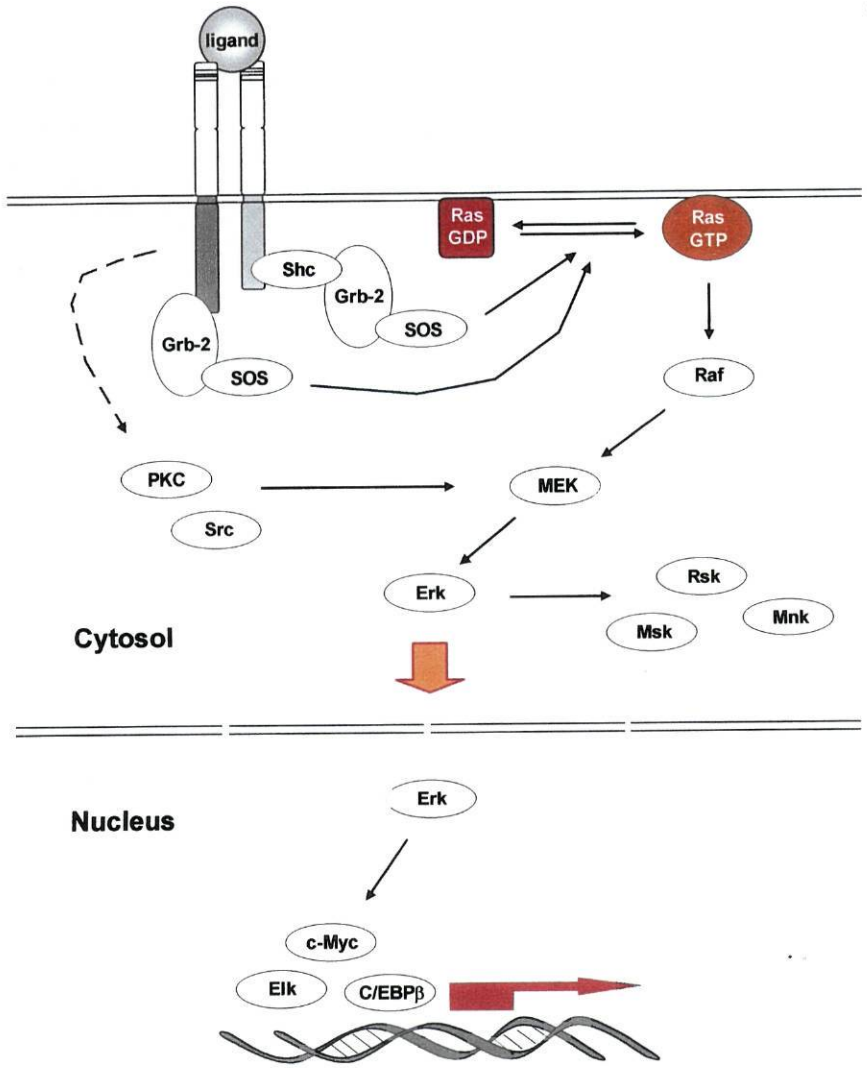

Figure 9. The Raf/MEK/Erk pathway. Ligand binding induces tyrosine phosphorylation of receptor cytoplasmic tail, providing docking sites for the adapter protein Shc. In turn, Shc recruits Grb-2 that associates with the Ras guanine nucleotide exchange factor SOS. Some receptors can also bind directly Grb-2 without the need for Shc. Exchange of GDP for GTP on Ras is catalyzed by SOS and leads to Ras activation. The MAPKKK Raf is consequently recruited to the membrane, phosphorylated and activated. PKC and Src can also activate Raf. Activated Raf phosphorylates MEK, which becomes activated and phosphorylates Erk, which becomes activated and phosphorylates several targets in the cytoplasm, including the kinases Rsk, Mnk and Msk. Subsequently, Erk translocates to the nucleus, where it phosphorylates and thereby modulates the activity of several transcription factors.

Erk1/2 Pathway in Viability, Proliferation and T-cell Differentiation

The multiplicity of downstream targets of Erk1/2 may partially explain some of the opposing effects associated with Raf/MEK/Erk pathway under different cellular contexts. For example, in fibroblasts Erk pathway has been shown to promote apoptosis [340], whereas in neuron cells it mediates cell survival via Rsk phosphorylation of Bad and CREB [121]. In other cell systems, Erk pathway has been associated predominantly with anti-apoptotic effects rather than cell death (e.g., Refs. [118,341,342]). Raf/MEK/Erk signals induce cell cycle progression by regulating the expression of c-Myc, cyclin D1, $p27^{kip1}$ and $p21^{cip1}$ [343]. However, the intensity and temporal pattern of activation of Erk pathway also appear to be relevant. While activation of constitutive low Raf signals induces cell proliferation, very high constitutive Raf signals originate cell cycle arrest [344]. It has further been suggested that intermediate-level activation of MEK/Erk is required for thymocyte differentiation from DP to SP stage, whereas high-level activation results in negative selection [345].

Erk pathway has a clear role in thymocyte differentiation, as evidenced by defective thymocyte maturation beyond the DP stage in Erk1-deficient mice [346], essentially by supporting positive selection [346-349] and favoring CD4 lineage commitment [342,348,350] but possibly also by setting up a threshold for negative selection through interaction with other pathways [345].

IL-7 and Erk1/2 Pathway

The effects of Erk/MAPK pathway on thymocyte differentiation are associated with TCR stimulation. IL-7 does not mediate Shc phosphorylation [351,352] or Raf/MEK/Erk pathway activation in human mature T-cells and murine T-cell lines [319,351,353,354]. Furthermore, activation of MEK/Erk appears to be unnecessary or redundant for proliferation induced by other γ_c-signaling cytokines [351]. However, it is important to stress that no studies were ever performed using normal primary thymocytes. Interestingly, Erk pathway appears to be important not only for positive selection of DP thymocytes, but also for development from DN to DP stage [348,355], a process in which IL-7 plays a critical role. Although at this maturational stage the signals activating MAPK are probably originated from the pre-TCR [355] it is not known whether these occur instead

of, or in combination with, IL-7-mediated stimulation. The absence of IL-7-induced MAPK activation is not consensual and some groups showed that IL-7 can activate Erk pathway in T and B lymphocytes [318,356], and induce Raf gene expression in thymocytes [261], raising the question of whether some of the effects of IL-7 during thymocyte development could be achieved through Raf/MEK/Erk pathway. The importance of this pathway for normal thymocyte development may also result from its intertwining with other critical pathways. For instance, Erk has been suggested to directly interact with and serine-phosphorylate STAT5, a major downstream target of IL-7, thereby contributing to STAT5 full activation [357]. Likewise, STAT3 serine phosphorylation in T-cells is dependent on MEK/Erk activity [358].

JNK/SAPK and p38^{MAPK} Pathways

IL-7 is capable of activating both JNK/SAPK and p38^{MAPK} pathways in human mature T-cells and murine T-cell lines [353], and at least p38^{MAPK} is required for transduction of IL-7-mediated proliferative signals [353], indicating that these pathways rather than being solely involved in stress responses can transduce mitogenic signals in T-cells. In fact, IL-15 and IL-7-induced proliferation of memory T-cells was shown to be dependent on p38^{MAPK} [359]. However, similar to Erk pathway, there are no reports regarding the effect of IL-7 stimulation upon JNK/SAPK and p38^{MAPK} pathways in primary thymocytes. Importantly, sustained activation of both pathways has been implicated in apoptosis [360,361], and negative selection of DP thymocytes [349,362]. Furthermore, constitutive activation of p38^{MAPK} pathway arrests cell cycle progression and completely blocks the differentiation of immature DN thymocytes [363]. These results implicate JNK/SAPK and p38^{MAPK} pathways as critical negative regulators of thymocyte development and mediators of apoptosis, rather than being involved in proliferative responses. In agreement with this view, a recent study using a mouse IL-7-dependent thymocyte cell line has shown that IL-7 withdrawal induces activation of JNK/SAPK and p38^{MAPK} , both of which are involved in cell death [354], possibly by mediating Bcl-2 downregulation and/or Bax intracellular localization [240,247,354]. Furthermore, activated p38^{MAPK} appears to directly phosphorylate the pH regulator NHE1, inducing a rapid intracellular alkalinization, disrupting mitochondrial metabolism and activating Bax [364].

The data regarding IL-7 connection to JNK/SAPK and $p38^{MAPK}$ pathways is scarce but suggest that these MAP kinases may be differentially activated and play distinct roles depending on the T-cell developmental stage.

PI3K/Akt(PKB) Pathway

PI3K and 3-phosphoinositide Phospholipids

Phosphatydilinositol-3-kinase (PI3K) is a fundamental regulator of different signaling pathways and numerous functions of the cell including proliferation and cell survival, differentiation, actin cytoskeleton reorganization, endocytosis, cytokine production and basic cellular metabolism, some of which might contribute to the involvement of PI3K in malignant transformation [365,366]. There are multiple PI3K isoforms that can be divided into three classes (reviewed in [365]). The forms linked to cytokine receptor signaling and lymphocyte activation belong to class I PI3Ks and consist of heterodimers with a 110 kD catalytic subunit (p110) and an adapter or regulatory subunit (p85) that contains several domains including one SH3 and two SH2 domains [366,367]. Although PI3K seemingly possesses protein serine/threonine kinase activity [368,369], most of PI3K effects are associated with its lipid kinase activity.

Upon activation, PI3K phosphorylates the D3 hydroxil position of the inositol ring of phosphatydilinositol or phosphoinositide (PI) phospholipids. Class I PI3Ks produce PI(3)P, $PI(3,4)P_2$, $PI(3,5)P_2$ and $PI(3,4,5)P_3$ *in vitro*. However, the major products in the cell appear to be $PI(3,4)P_2$, and especially $PI(3,4,5)P_3$ [365,370]. A significant number of proteins have been shown to interact with PI3K and/or its D3-phosphorylated PI products [366], but none has been so deeply studied and shown to play such a critical role in transduction of PI3K-mediated signals as the serine/threonine protein kinase B (PKB), also known as Akt [371-374].

Multistep Activation of Akt/PKB by PI3K

Activation of Akt/PKB is a multistep process. Receptor stimulation of PI3K leads to production of $PI(3,4,5)P_3$, which recruits inactive cytosolic Akt/PKB to the plasma membrane by high affinity binding to Akt/PKB pleckstrin homology (PH) domain [375-377]. Binding of Akt/PKB to the membrane appears to result in conformational changes [375,377]

and formation of homomultimers that may increase Akt/PKB intrinsic catalytic activity [378]. Akt/PKB has three regions: an N-terminal PH domain, a kinase domain and a C-terminal regulatory tail [365]. For Akt/PKB to become fully activated it must subsequently be phosphorylated at two key residues, threonine 308 (T308) in the kinase domain activation loop and serine 473 (S473) in the regulatory domain. Akt/PKB phosphorylation at T308 is catalyzed by the ubiquitously expressed 3-phosphoinositide-dependent protein kinase 1 (PDK1). PDK1 itself is targeted to the membrane through its PH domain as a consequence of PI3K activation [379,380]. The kinase responsible for S473 phosphorylation remains to be clearly identified, but has been named PDK2. Different reports suggested that integrin-linked kinase (ILK) [381,382], PDK1 modified by complex formation with PKC-related kinase 2 (PRK2) or a similar protein [383], or even partially activated Akt/PKB itself [384], could be associated with PDK2 activity. After full activation, Akt/PKB translocates from the plasma membrane to the cytosol and the nucleus by as yet unknown mechanisms that may include interaction with other proteins such as the protooncogene Tcl1 [385], whose activation is the hallmark of T-cell prolymphocytic leukemia. Akt/PKB nuclear localization may have the dual role of allowing the access to target proteins and controlling its cytoplasmic activity by sequestration in the nucleus [386].

Lipid Phosphatases: Negative Regulators of PI3K/Akt Pathway

The activity of Akt/PKB is negatively regulated by lipid phosphatases. The tumor suppressor PTEN (phosphatase and tensin homologue deleted from chromosome 10) or MMAC (mutated on multiple sites in advanced cancers) is a weak protein phosphatase and a potent lipid phosphatase [387,388] that antagonizes PI3K action by dephosphorylating D3-phosphorylated PIs, thereby preventing Akt/PKB activation. Somatic deletions or mutations involving PTEN are commonly found in human cancers [389,390]. The crucial role of PTEN as a tumor suppressor has been further stressed by gene targeting in mouse models. PTEN -/- mice are embryonic lethal [391] and exhibit stem cells with increased *in vivo* and *in vitro* proliferation, impaired ability to differentiate and decreased susceptibility to a variety of apoptotic stimuli [392,393], whereas mice heterozygous for PTEN-inactivating mutations have a high tendency to develop malignancies of various

origins, including T-cell lymphomas, and demonstrate impaired Fas-induced apoptosis [394]. Moreover, cells from PTEN +/- and -/- mice have elevated levels of phosphorylated Akt/PKB and increased proliferation [393]. While overexpression of PTEN in some tumor cell lines causes cell cycle arrest and apoptosis [388,390,395], the decrease in apoptotic sensitivity resulting from PTEN deficiency can be opposed by overexpression of dominant negative forms of Akt/PKB [390] or by inhibiting the action of Akt/PKB downstream targets [396], which demonstrates the negative association between PTEN and PI3K/Akt(PKB) pathway. Furthermore, this inverse correlation has been observed in human hematological malignancies [397]. Another lipid phosphatase that can downmodulate Akt/PKB activity is SHIP (SH2-containing inositol 5-phosphatase), which catalyzes the hydrolysis reaction that originates $PI(3,4)P_2$ from $PI(3,4,5)P_3$. Cells from SHIP -/- mice show prolonged activation of Akt/PKB upon stimulation and have impaired apoptotic responses. The myeloid lineages from these mice exhibit increased cell survival [398]. In addition, overexpression of SHIP was shown to inhibit Akt/PKB activation and to induce apoptosis of lymphoid cells [399]. Negative regulation of Akt/PKB is further achieved through dephosphorylation of T308 and S473, possibly by the serine/threonine phosphatase 2A (PP2A) [386,400,401]. PP2A has also been shown to dephosphorylate and consequently promote the pro-apoptotic function of Bad [402]. Since Bad is inactivated and targeted for degradation through Akt/PKB-mediated phosphorylation [119,120], this potentially defines PP2A as a negative regulator of the PI3K/Akt(PKB) pathway at different levels, a possibility that correlates well with the hypothesis that PP1 and PP2A are tumor suppressors [140].

PI3K/Akt Pathway in Cell Viability, Proliferation and Cancer

PI3K/Akt(PKB) pathway has been consistently and systematically associated with prevention of apoptosis, and overexpression of Akt/PKB prevents or delays cell death in diverse cellular model systems [340,376,403-407]. This role of Akt/PKB together with its clear capacity to induce cell cycle progression [393,395,408,409], is certainly of significant relevance for PI3K and Akt/PKB involvement in cancer progression [389,410,411].

Prevention of cell death by Akt/PKB has been linked to direct inhibition of pro-apoptotic molecules. Bad is phosphorylated by Akt/PKB at serine 136 [119,120].

Phosphorylated Bad is sequestered in the cytosol by 14-3-3 proteins. This neutralizes Bad apoptotic effect by preventing heterodimerization with Bcl-x_L and Bcl-2 [412]. Caspase 9 can also be phosphorylated and inhibited by Akt/PKB [413], although it is not certain how relevant and common this mechanism is for Akt/PKB inhibition of apoptosis in different cell systems [365] (Figure 10). Akt/PKB has further been shown to maintain mitochondria integrity and prevent the release of cytochrome c by blocking Bax-, Bid- and Bim-mediated cell death [414,415].

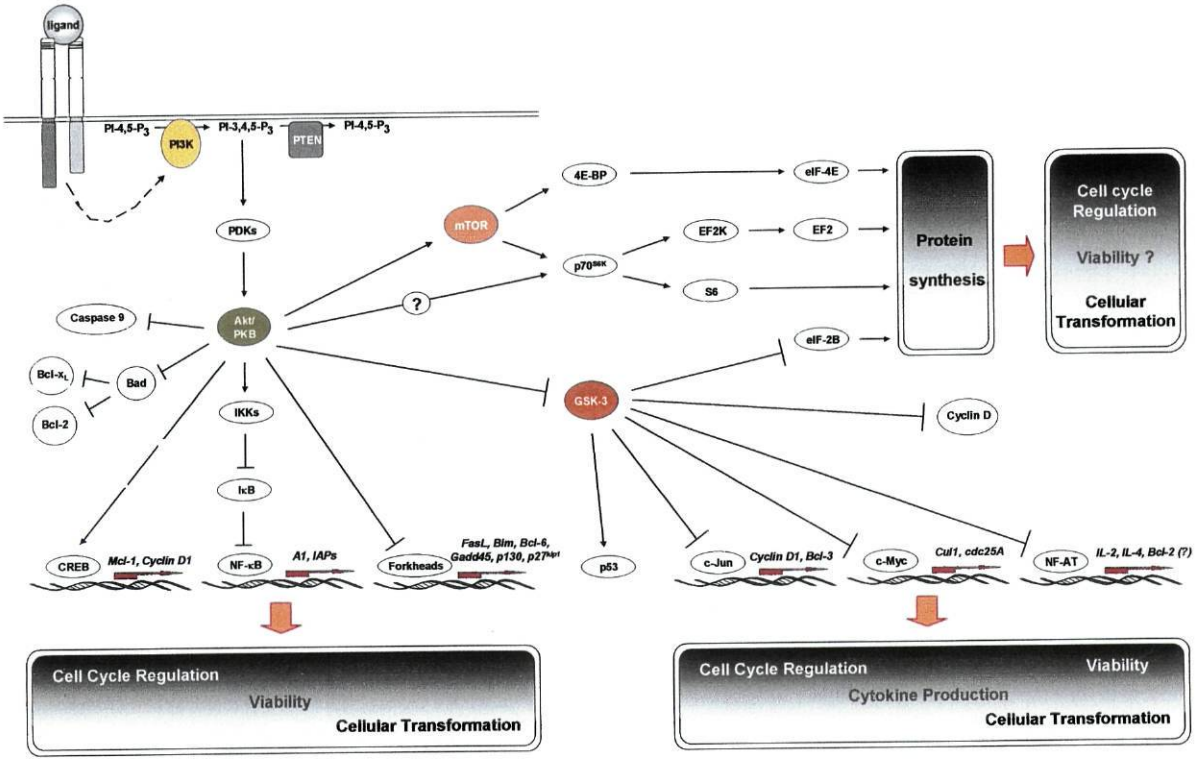

Figure 10. The PI3K/Akt pathway. Receptor-activated PI3K mediates the phosphorylation of PI-4,5-P_2 into PI-3,4,5-P_3, thereby recruiting PDK and Akt/PKB to the membrane, and inducing Akt activation by PDK. The effect of PI3K is opposed mainly by the phosphatase PTEN. Activated Akt phosphorylates and thus modulates the activity of numerous targets that are directly or indirectly involved in regulation of protein synthesis, cell cycle progression, and viability, as well as cell-specific functions such as cytokine production. Over-activation of PI3K/Akt pathway is involved in tumorigenesis. See text for detailed explanation.

Regulation of Gene Transcription

Akt/PKB regulation of cell death and cell proliferation is also accomplished via control of gene transcription and protein synthesis. Transcription factors of the Forkhead family (FKHR/FoxO1, FKHRL1/FoxO3 and AFX/FoxO4) are directly phosphorylated by Akt/PKB [416-420]. Phosphorylation promotes their export from the nucleus and retention in the cytosol by interaction with 14-3-3 proteins, which ultimately prevents activation of their target genes [419,420]. Transcription targets for the Forkhead family members include the pro-apoptotic genes FasL [420] and Bim [421,422], as well as the cell cycle inhibitor $p27^{kip1}$ [160,161,422], the retinoblastoma-like protein p130 [423], the transcriptional repressor Bcl-6 that downregulates the expression of the anti-apoptotic gene Bcl-x_L [424], and the DNA damage response gene Gadd45 that mediates G2/M cell cycle arrest and DNA repair [425,426]. Thus, Akt/PKB appears to contribute to cell survival, cell cycle progression and cellular transformation by phosphorylating and inhibiting transcription of critical genes by Forkheads [427]. Interestingly, PI3K/Akt(PKB) pathway was also suggested to be involved in regulation of $p27^{kip1}$ protein stability [428], and Akt/PKB was recently shown to be able to directly phosphorylate $p27^{kip1}$ at serine 10, threonine 187 and the newly identified target residue threonine 198, therefore promoting $p27^{kip1}$ binding to 14-3-3 proteins, cytoplasmic localization and degradation by the ubiquitin-proteasome pathway [170]. This means that the PI3K/Akt(PKB) pathway is able to regulate the expression of this crucial cell cycle inhibitor by both transcriptional and post-transcriptional mechanisms. Since evidence is building up that implicates $p27^{kip1}$ in induction of apoptosis [161,429,430], hence contributing to $p27^{kip1}$ classification as a tumor suppressor, this further stresses the importance of PI3K/Akt(PKB) pathway in cancer progression.

Transcription factors that are indirectly regulated by Akt/PKB include CREB, E2F and NF-κB. Akt/PKB mediates phosphorylation of CREB, consequently inducing transcription of CREB targets [431] that include anti-apoptotic genes such as Mcl-1 [432]. Accordingly, Mcl-1 expression is upregulated independently via PI3K/Akt and MEK/Erk pathways in response to cytokine stimulation [118], and both pathways have been shown to activate CREB [121,431]. Activated Akt/PKB induces Rb phosphorylation and E2F activity by upregulating cyclin D3 and downregulating $p27^{kip1}$ [409]. Akt/PKB can also induce the activation of NF-κB by binding to and activating I-κB kinases (IKKs) [125,433,434]. NF-κB is

inhibited when associated with I-κB. Phosphorylation of I-κB by IKKs targets the NF-κB inhibitor for degradation, thereby allowing NF-κB to exert its function and promote transcription of target genes, including those involved in protection from apoptosis (e.g. pro-survival Bcl-2 family member A1 and caspase inhibitors IAP1 and IAP2) [124,125,434,435].

Regulation of Protein Synthesis

The role of Akt/PKB on protein synthesis is achieved by modulation of the activity of several factors that are required for mRNA translation and ultimately regulate the expression of proteins that affect cell cycle and survival. Akt/PKB appears to directly phosphorylate mTOR (mammalian target of rapamycin; also known as FRAP or RAFT) that in turn phosphorylates and activates S6 kinase ($p70^{S6K}$) [390,436-439]. It is debated whether Akt/PKB itself may also directly phosphorylate $p70^{S6K}$ [373,390]. Both $p70^{S6K}$ and mTOR indirectly control protein synthesis. Phosphorylation of the repressor of translation 4E-BP (eIF-4E binding protein) by mTOR, allows for eIF-4E dissociation from 4E-BP and consequent activation of mRNA translation [440-442]. $p70^{S6K}$ has two physiological targets, the S6 ribosomal protein and EF2 kinase. Phosphorylation of S6 enhances the biosynthesis of ribosomal proteins and elongation factors, whereas phosphorylation of EF2 kinase permits the activation by dephosphorylation of elongation factor-2 (EF2), therefore contributing to protein synthesis [443-445]. Importantly, $p70^{S6K}$ and mTOR were also shown to be critical for G1 to S-phase progression [446,447]. The relevance of PI3K/Akt(PKB)/mTOR/$p70^{S6K}$ in cellular transformation has recently been underlined by several reports demonstrating that inhibition of this pathway prevents tumorigenesis both *in vitro* and *in vivo* [396,448,449].

The Multiple Roles of GSK-3

Akt/PKB stimulates protein synthesis by phosphorylating and inhibiting another important downstream target, glycogen synthase kinase 3 (GSK-3). Inhibition of GSK-3 by Akt/PKB increases mRNA translation via activation of the guanine nucleotide exchange factor eIF-2B that is required for initiation of protein synthesis [450]. GSK-3 is a highly conserved serine/threonine kinase that has been reported to possess also tyrosine

kinase activity [451] and was first identified as a key negative regulator of glycogen synthase enzyme that is involved in glycogen synthesis [452]. Two related genes have been cloned in vertebrates, GSK-3α and GSK-3β [453]. GSK-3 has a high basal activity within resting cells. Stimulation of PI3K/Akt(PKB) pathway leads to direct phosphorylation of GSK-3α (at serine 21) and GSK-3β (at serine 9) by Akt/PKB. Phosphorylation by Akt/PKB inhibits GSK-3 activity [454] and plays an important role in survival signaling. Overexpression of constitutively active GSK-3 originates apoptosis, whereas dominant negative GSK-3 prevents apoptosis induced by PI3K/Akt(PKB) inhibition [455,456], indicating that abrogation of GSK-3 activity is a critical step in Akt/PKB survival pathway. However, the mechanism by which GSK-3 regulates cell survival is not known. One possibility is that GSK-3 contributes to p53-dependent apoptosis. PI3K and Akt/PKB significantly delay the onset of p53-mediated, transcriptionally-dependent cell death [407], and recently GSK-3β has been reported to phosphorylate p53 at serine 33 *in vitro* and increase the transcriptional activity of p53 *in vivo* [457]. Moreover, GSK-3β regulation of p53 was shown not to be involved in DNA damage response [457], suggesting that GSK-3β could provide the link between PI3K/Akt(PKB) and non-DNA damage (i.e., oncogene activation or growth factor withdrawal) p53-mediated apoptosis. Curiously, activation of PI3K/Akt(PKB) can also override p53-independent DNA damage-induced cell cycle arrest of hematopoietic cells [458], possibly via inactivation of Forkhead transcription factors [425,426].

Besides regulating protein synthesis and cell survival, GSK-3 modulates the activity and nuclear localization of several molecules that have been shown to stimulate cell cycle progression. GSK-3β phosphorylates cyclin D1, promoting its nuclear export and proteasomal degradation [459-461], a process that appears to be essential for normal cell division, since its deregulation results in cellular transformation [460]. Jun family of transcription factors, c-Jun in particular but also JunB and JunD, are phosphorylated and inhibited by GSK-3 both *in vitro* and *in vivo* [462-464]. GSK-3 phosphorylation of c-Myc at threonine 58 accelerates c-Myc protein degradation [336,465]. Nuclear factor of activated T-cells (NF-ATc), another transcription factor involved in proliferation, is also phosphorylated by GSK-3. This results in inhibition of NF-ATc ability to bind DNA and enhancement of its nuclear export, which physically impedes transcription of target genes

[466-468]. In *Dictyostelium* a GSK-3 homologue has been shown to promote nuclear export of STATs [469], an event that so far has not been reported in mammalian cells and deserves investigation. The PI3K/Akt(PKB) pathway is summarized in Figure 10.

PI3K/Akt Pathway in Normal T-cell Biology and Leukemogenesis

The characterization of PI3K/Akt(PKB) in the previous sections was based on available information that primarily results from experiments performed with cell models other than T lymphocytes. Thus, the question arises of what is the actual role of this pathway on T-cell biology. As expected, PI3K/Akt(PKB) pathway appears to promote both cell cycle progression and survival of T-cells [408,409,470,471]. Akt/PKB negatively regulates the proapoptotic function of Bad in primary T-cells [472] and activates NF-κB anti-apoptotic transcription factor in Jurkat T-cells [125]. Akt/PKB phosphorylates and hence inhibits GSK-3 in T-cells [473], whereas GSK-3 negatively regulates T-cell proliferation and IL-2 production, in part by inducing NF-ATc nuclear export [467]. Since PI3K was shown to be required for NF-AT transcriptional activity and IL-2 production following TCR-mediated activation [474], it seems that PI3K induces NF-AT activation of IL-2 transcription via Akt/PKB-mediated inhibition of GSK-3, which indirectly leads to cell proliferation. Importantly, since GSK-3 inactivation has an impact on several downstream targets it will be interesting to evaluate which ones are involved in the proliferation and viability of T-cells.

Expression of active Akt/PKB in T-cells of transgenic mice was shown to inhibit Fas-mediated T-cell death and to induce a profound lymphoproliferative disorder with multiorgan infiltration, demonstrating that PI3K/Akt(PKB) pathway can contribute to T-cell homeostasis *in vivo* [475]. Transgenic mice expressing an activated form of PI3K under the control of the Lck promoter present a similar phenotype [476]. In agreement with a role for PI3K/Akt(PKB) in regulation of death receptor-induced apoptosis [477], Akt/PKB has been shown to increase the expression of cFLIP, a molecule that, as mentioned above, is known to inhibit Fas-induced cell death [478]. Furthermore, T-cells from PTEN +/- mice show impaired Fas-mediated apoptosis, reduced activation-induced cell death, increased proliferation and autoimmunity [394], whereas mice with T-cell-specific deletion of PTEN present increased thymocyte and mature T-cell numbers, spontaneously activated T-cells,

impaired tolerance and reduced T-cell apoptosis. Notably, these mice die from the occurrence of T-cell lymphomas [479]. Also remarkable is the fact that Jurkat and other T-cell leukemia cell lines lack expression of PTEN and/or SHIP, and consequently have high PI3K and Akt/PKB basal activities [480,481], suggesting that overactivation of PI3K/Akt(PKB) pathway could be a general feature of T-leukemia cell lines and raising the question of whether this reflects a defect in primary T-ALL cells. Altogether these studies reveal the critical importance of the balance between the activities of PI3K/Akt(PKB) and PTEN in regulating normal T-cell development, activation and death, and underscore the consequences of destroying that balance for the progression of cancer and autoimmunity.

In mature T-cells, IL-2 and TCR stimulation have been shown to activate PKB/Akt and $p70^{S6K}$ in a PI3K-dependent way [473,482]. PI3K and Akt/PKB were also reported to be involved in CD28-mediated T-cell costimulation [483-486] and CD5 signaling in mature T-cells [487].

IL-7 and PI3K/Akt Pathway

IL-7 is known to induce activation of PI3K and PI(3,4,5)P3 production [224,294,295,488], leading to proliferation [294] and survival of T-cells [325]. However, the mechanisms through which IL-7 exerts its effects via PI3K in T-cells are still being dissected. Activation of PI3K by IL-7 was initially shown to be dependent on tyrosine phosphorylation [488] and subsequently on Jak3 tyrosine kinase activity [294]. Jak3 associates with the p85 regulatory subunit of PI3K in IL-7 stimulated T-cells and appears to regulate PI3K activation by tyrosine-phosphorylating p85 [294]. The IL-7R activates PI3K by at least two routes. The cytoplasmic tail of IL-7Rα contains a motif that is phosphorylated at a tyrosine residue after activation, which binds PI3K through its SH2 domain and activates the p110 catalytic subunit [489]. In addition, IL-7R mediates recruitment and phosphorylation of insulin receptor substrate-1 (IRS-1) and IRS-2, which are associated with (and possibly phosphorylated by) Jak1 and Jak3. Phosphorylated IRS-1/2 appear to recruit and activate PI3K [490,491]. Activation of PI3K is apparently indispensable for IL-7-induced proliferation [294]. The role of PI3K on IL-7-mediated viability of T-cells is somewhat more controversial. Although it is generally accepted that PI3K/Akt(PKB)

pathway is associated with the anti-apoptotic effects of IL-7 and other γ_c-signaling cytokines [492], one study indicated that PI3K is dispensable for the trophic action of IL-7 on murine immature DN thymocytes [240]. On the contrary, others suggest that PI3K/Akt(PKB) is critical for survival and proliferation of human T-cell precursors [325]. This discrepancy might be the consequence of differences between the two species or the culture methods used. In any case, given the conserved role of PI3K/Akt(PKB) on regulation of cell viability one would expect this pathway to be involved, to greater or lesser extent, in IL-7-mediated survival of thymocytes and mature T-cells. In fact, there is plenty indirect evidence that this is the case. IL-7 has been shown to upregulate IL-2 gene expression in activated human T lymphocytes by enhancing the transcriptional activities of NF-AT and activator protein-1 (AP-1) [267], an effect that has been shown to be mediated by PI3K and Akt/PKB in response to IL-2 and TCR stimulation [467,474]. NF-AT and AP-1 were both suggested to mediate survival by regulating Bcl-2 gene transcription [493,494]. Recently, transcription factors of the Forkhead family were shown to be phosphorylated and inactivated by IL-2 in a PI3K-dependent manner, and their inactivation played a critical role not only in proliferation but also survival of T-cells [422]. This strengthens the possibility that PI3K/Akt(PKB) pathway might accomplish the same function in IL-7 signaling. Another hint that PI3K and Akt/PKB play a role in IL-7-mediated viability comes from the knowledge that IL-7 regulates the expression of c-myb gene in cutaneous T-cell lymphoma cells [495]. c-myb is an important transcriptional regulator of proliferation and survival, and c-myb expression in T-cells is regulated by Akt/PKB after IL-2 stimulation [471]. Most importantly, γ_c-cytokines have recently been shown to promote T-cell survival by activating Akt/PKB [496].

Chapter 2

CYTOKINES THAT SIGNAL THROUGH THE γ_C-CHAIN INDUCE PROLIFERATION OF T-ALL CELLS

João T. Barata, Angelo A. Cardoso, Thomas D. Keenan, Lee M. Nadler, Vassiliki A. Boussiotis

In preparation

Abstract

Common gamma chain (γ_c)-signaling cytokines, IL-2, IL-4, IL-7, IL-9 and IL-15 have distinct effects on T-cell development, survival, proliferation and differentiation, and have been directly or indirectly associated with T-cell leukemogenesis. Surprisingly, with the exception of IL-2 and IL-7, little is known about the effects of these cytokines on T-cell acute lymphoblastic leukemia (T-ALL). Here, we showed that all γ_c-signaling cytokines have the ability to promote proliferation of primary T-ALL cells. IL-7 induced the most robust proliferative responses and was the cytokine to which a higher number of patients were responsive. IL-4 preferentially stimulated the proliferation of samples with a more mature immunophenotype, whereas CD1a-positive cortical T-ALL cells were less responsive to IL-9 than cells from other stages of maturation. Our studies also showed that combinations of two γ_c-signaling cytokines could have synergistic or additive proliferative effects. Proliferation induced by γ_c-signaling cytokines was mediated through calcium-independent pathways, and IL-7-mediated proliferation was dependent on Jak3 activation, as demonstrated by the use of a Jak3-specific inhibitor. Overall, our studies demonstrated that γ_c-dependent cytokines are capable of mediating proliferation of T-ALL cells and raised the possibility of synergistic or additive effects existing *in vivo*. IL-9 and IL-15 are here implicated for the first time as potentially involved in the biology of the disease. Since IL-7 mediates the most striking proliferative effect and has been shown to act as an oncogene in the living organism, our studies support the hypothesis that IL-7 may function as a critical regulator of T-ALL *in vivo*.

Introduction

IL-2, IL-4, IL-7, IL-9 and IL-15 belong to a family of cytokines whose receptors share the gamma-common (γ_c) chain. Despite significant redundancy, these γ_c-signaling cytokines have essentially distinct and often critical effects in normal T-cell development, survival, proliferation and differentiation. Importantly, they have also been directly or indirectly implicated in T-cell leukemogenesis. Numerous studies have demonstrated that IL-7 is not only critical for normal thymocyte development (reviewed in Ref. [1]), but might also contribute to development of T-cell leukemia [2-5]. Evidence implicating other γ_c-signaling cytokines in thymocyte development and/or T-cell leukemogenesis is more controversial. IL-2 has been suggested to act as a negative regulator during distinct phases of T-cell development in the thymus [6-9]. In contrast, several studies suggest that IL-2 might have a positive effect in the progression of T-cell leukemia. T-ALL cells express the receptors for IL-2 [5,10-12], appear to be able to secrete functional IL-2 [13], and proliferate in response to the cytokine [2,11-15], raising the possibility of an IL-2 autocrine loop existing in some T-ALL cases. In contrast to IL-2 and IL-7, not much evidence has been gathered regarding the involvement of IL-4 in T-ALL. Nonetheless, IL-4 was shown to mediate T-ALL proliferation [2,15], and survival [5]. To our best knowledge, no studies have been published on the effect of IL-9 or IL-15 in T-ALL, although it has been demonstrated that IL-9 is a major *in vitro* anti-apoptotic [16] and growth factor [17] for thymic T-cell lymphomas, and IL-15 transgenic mice develop fatal lymphocytic leukemia (with a T-NK phenotype) [18].

In the present study, we showed that IL-2, IL-4, IL-7, IL-9 and IL-15 induce proliferation of primary T-ALL cells and can have synergistic effects when used in combination. IL-9 and IL-15 are for the first time implicated in T-ALL cell expansion. Importantly, we showed that IL-7 is the γ_c-signaling cytokine that mediates a proliferative effect most frequently and more dramatically. This, together with the fact that IL-7 is produced in the microenvironment where the leukemia arises, suggests that IL-7 might play a critical role in the biology of T-ALL.

Materials and Methods

Primary T-ALL samples. T-ALL cells were obtained from the peripheral blood and/or the bone marrow of patients with high leukemia involvement (85-100%). Informed consent and Institutional Review Board approval was obtained for all sample collections. Samples were enriched by density centrifugation over Ficoll-Hypaque and then washed twice in RPMI-1640 supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 2 mM L-glutamine (further referred to as RPMI-10).

Immunophenotype. Expression of cell surface molecules was determined by direct labeling using standard methodology [19]. Fc receptors were blocked by incubation with mouse Ig before the addition of the specific monoclonal antibodies (mAbs). The mAbs used were fluorescein isothiocyanate (FITC)-conjugated anti-CD3 and CD2 and phycoerythrin (PE)-conjugated anti-CD1a, CD4, CD5, CD7, CD8, CD19, CD13, CD14, CD33 and CD34. Irrelevant isotype-matched antibodies were used as negative controls. Samples were acquired in a Coulter Elite, Coulter XL (Beckman-Coulter, Miami, FL) or FACSCalibur (Beckton-Dickinson, San Jose, California) flow cytometer, and stored in listmode files. At least 5,000 gated events were measured for each sample. For the expression of cytoplasmic CD3, cells were incubated with unconjugated anti-CD3 antibody for 20 minutes at 4°C to ensure that potential surface expression of CD3 did not affect the assessment of intracellular CD3. Cells were then washed in PBS and fixed in 0.1 % formaldehyde for 30 minutes at 4°C, washed in PBS and resuspended in 1x Perm/Wash Solution (Pharmingen, San Diego, CA), before adding FITC-conjugated anti-CD3 mAb (Pharmingen).

Proliferation assay. Cells ($2x10^6$ cells/ml) were cultured in triplicates in RPMI supplemented with 10% FBS (RPMI-10), in flat-bottom 96-well plates at 37°C with 5% CO_2. Cultures were carried for 72 or 96 hours, in media without cytokine (control condition), a single cytokine or combinations of two of the following cytokines: IL-2 (100 U/ml), IL-4 (10 ng/ml), IL-7 (10 ng/ml), IL-9 (50 U/ml), or IL-15 (20 ng/ml). When indicated, the Jak3-specific inhibitor WHI-P131 (50μM; Calbiochem, San Diego, CA)

was added to the cultures with IL-7. Cells were incubated with ^{3}H-Thymidine (1 μCi/well) for 16 hours prior to harvest. DNA synthesis, as measured by ^{3}H-Thymidine incorporation, was assessed using a liquid scintillation counter. Proliferation index (PI) was calculated as: PI = [cpm experimental condition / cpm control condition]. A cytokine was considered to induce proliferation when the PI was at least 1.5 and the mean of the triplicates was higher than 2 standard deviations of the control.

Calcium flux assay. For the calcium flux experiments, Indo-1 AM (Molecular Probes, Eugene, OR) was used as fluorescent Ca^{++} indicator following the manufacturer's instructions. Briefly, primary T-ALL cells were loaded with Indo-1 for 45 minutes at room temperature. After 2 washes with PBS, cells were resuspended in AIM V medium for flow cytometry analysis using a Coulter Elite flow cytometer. Cytokines were added after 30 seconds of acquisition at the following concentrations: 500 U/ml IL-2, 50ng/ml IL-4, 50 ng/ml IL-7, 200 U/ml IL-9 and 100 ng/ml IL-15. Events were acquired for a total of 5 minutes. Both negative (medium alone) and positive (Ionomycin) controls were used.

Statistical Analysis. Differences between the proliferative effect of cytokines were assessed using the nonparametric Wilcoxon signed ranks test. Differences between two (e.g. CD1+ *versus* CD1- samples) or more (e.g. EGIL classification stages) independent variables (i.e. populations) regarding the degree of proliferation to each cytokine or the intensity of expression of a certain marker, were evaluated using the 2-tailed nonparametric Mann-Whitney test and the nonparametric Kruskal-Wallis test, respectively. Differences were considered significant for $P<0.05$.

Results

All γ_c-signaling cytokines mediate proliferation of T-ALL cells

Primary T-ALL cells were enriched by density centrifugation, immunophenotyped and classified according to their maturation stage using the criteria defined by Uckun et al. [20] or the European Group for Immunological Characterization of Leukemias (EGIL) [21] (Table 1). As shown in Figure 1 for three representative patients, all samples expressed high levels of cytoplasmic CD3, confirming their T-cell lineage phenotype. No aberrant expression of the lineage markers CD19 (B-cell), CD13, CD14 or CD33 (myeloid) or CD56 (NK cell) was detected with the exception of one patient (T-ALL# 13), whose leukemic cells expressed the myeloid marker CD33 (Table 1). Five cases showed positivity for the common ALL antigen CD10, a molecule that is transiently expressed at the early cortical stages of thymic differentiation, being downregulated as CD3 expression increases [22,23]. In accordance, four of the five CD10-positive cases did not express surface CD3.

Primary T-ALL cells were cultured *in vitro* for 72 and/or 96 hours in medium alone or in the presence of IL-2, IL-4, IL-7, IL-9 or IL-15. Proliferation was assessed by ^{3}H-thymidine incorporation. A cytokine was considered to induce proliferation when the proliferation index was at least 1.5 and the mean of the triplicates was higher than 2 standard deviations of the control. Although we used T-ALL samples with high leukemia content, the possibility existed that the cells proliferating in response to the γ_c-signaling cytokines were residual normal T-cells. To confirm the malignant origin of the proliferating cells, two alternative approaches were undertaken: 1) S+G_2/M cycling cells were isolated by cell sorting and were evaluated by PCR analysis using patient-specific primers for the presence of specific clonal TCR rearrangement(s) identified at diagnosis (see Chapter 3); 2) the immunophenotype of unstimulated primary cells was compared to that of cells sorted or gated at S+G2/M after 72 hours of cytokine stimulation (e.g., for the presence of surface CD3- T-ALL cells; data not shown). Both approaches indicated that the cytokine-responsive proliferating cells were leukemic T-cells.

Table 1. Immunophenotype and classification of T-ALL samples

T-ALL #	Immunophenotype													Maturation Stage	
	CD1	CD2	CD3	CD4	CD5	CD7	CD8	CD10	CD13 CD14	CD19	CD33	CD34	CD56	Uckun et al.	EGIL
1	-	+	-	-	+	+	-	-	-	-	-	-	-	II	II
2	+	+	+	-	+	+	-	-	-	-	-	+	-	III	III
3	-	+	+	+	+	+	+	-	-	-	-	nd	-	III	IV
4	+	+	-	+	+	+	+	-	-	-	-	-	-	II	III
5	-	+	+	-	+	+	-	-	-	-	-	+	-	III	IV
6	+	+	+	-	+	+	-	-	-	-	-	-	-	III	III
7	+	-	+	+	+	+	-	-	-	-	-	-	-	III	III
8	-	+	-	+	+	+	-	+	-	-	-	+	-	II	II
9	+	+	-	+	+	+	+	-	nd	nd	-	-	nd	II	III
10	+	+	+	nd	+	+	+	-	-	-	-	-	-	III	III
11	-	+	+	-	+	+	+	-	-	-	-	-	-	III	IV
12	-	+	+	-	+	+	+	-	-	-	-	-	-	III	IV
13	-	-	-	+	+	+	-	-	-	-	+	+	-	II	II
14	+	+	+	-	+	+	-	-	-	-	-	-	-	III	III
15	-	+	+	+	+	+	-	-	-	-	-	-	-	III	IV
16	+	-	-	+	+	+	-	+	-	-	-	-	-	II	III
17	+	+	-	-	+	+	+	+	-	-	-	-	-	II	III
18	-	+	+	-	+	+	-	-	-	-	-	-	-	III	IV
19	-	+	-	+	+	+	-	+	-	-	-	+	-	II	II
20	+	+	+	+	+	+	+	-	-	nd	-	-	-	III	III
21	+	+	-	+	+	+	+	-	-	-	-	+	-	II	III
22	-	+	-	+	+	+	+	-	-	-	-	-	-	II	II
23	-	+	+	-	+	+	-	-	-	-	-	+	-	III	IV
24	-	+	+	+	+	+	-	-	-	-	-	+	-	III	IV
25	+	+	+	-	+	+	+	+	-	-	nd	-	-	III	III

Immunophenotype: +, at least 30% cells stained above the isotype control; -, less than 30% stained above the isotype control; nd, not determined. T-cell maturation stages were defined as described (Refs. [20,21]). Classification by Uckun et al.: Stage I (Pro-TL): CD7+ CD2- CD5- CD3-; Stage II (Immature TL): CD7+ (CD2 and/or CD5)+ CD3-; Stage III (Mature TL): CD7+ CD2+ CD5+ CD3+. Classification by EGIL: Stage I (Pro-T-ALL): CD7+ CD2- CD5- CD8-; Stage II (Pre-T-ALL): CD7+ (CD2 and/or CD5 and/or CD8)+; Stage III (Cortical T-ALL): CD1+; Stage IV (Mature T-ALL): CD1-CD3+. T-ALL patients were numbered 1-25 (T-ALL#).

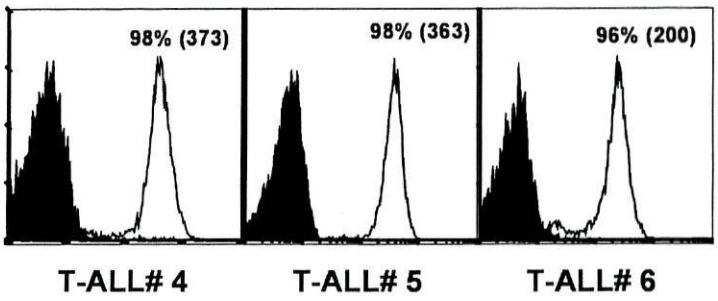

Figure 1. T-ALL cells express cytoplasmic CD3. Primary T-ALL samples were immunophenotyped for expression of intracellular CD3 (cCD3). Percentage of positive cells as compared to isotype control (black histograms) and mean intensity of fluorescence for cCD3 expression (in brackets) are indicated. Results from 3 representative patients are shown.

All the γ_c-signaling cytokines induced proliferation of T-ALL cells, although a considerable inter-patient variation was observed (Table 2). IL-7 was the cytokine that stimulated a higher number of patients (18 of 25; 72%), followed by IL-4 (64% of the cases). IL-2 and IL-9 induced proliferation in respectively 44% and 40% of the patients, whereas IL-15 only stimulated responses in 24% of the patients. Only 2 patients (8%) were completely unresponsive to all the cytokines tested. It is noteworthy that the majority of the patients (64%) showed a proliferative response to at least 2 different cytokines, and IL-7 was one of the stimulatory cytokines in all those cases (16 of 16 cases). None of the patients responded exclusively to IL-2, IL-9 or IL-15.

Table 2. Proliferation of T-ALL samples in response to γ_c-signaling cytokines

T-ALL #	Maturation Stage		Proliferative response to cytokine				
	Uckun et al.	EGIL	IL-2	IL-4	IL-7	IL-9	IL-15
1	II	II	■		■	■	■
2	III	III	■	■	■	■	■
3	III	IV		■			
4	II	III			■		
5	III	IV	■	■	■	■	■
6	III	III		■	■		
7	III	III		■			
8	II	II	■		■	■	
9	II	III	■	■	■		■
10	III	III		■			
11	III	IV		■			
12	III	IV		■			
13	II	II	■		■	■	
14	III	III			■		
15	III	IV	■	■	■	■	■
16	II	III					
17	II	III		■	■		
18	III	IV	■	■	■	■	
19	II	II	■	■	■	■	■
20	III	III		■	■		
21	II	III	■	■	■		
22	II	II		■	■		
23	III	IV		■	■	■	
24	III	IV	■				
25	III	III					
Number of responsive patients			11	16	18	10	6
Percentage of responsive patients			44	64	72	40	24

Primary T-ALL cells were cultured in medium alone or with cytokines and proliferation was assessed after 72 or 96 hours of culture, as described in "Materials and Methods". A cytokine was considered to induce proliferation when the proliferation index (PI) was at least 1.5 and the increase in proliferation in comparison to the control was statistically significant (see "Materials and Methods"). Black squares (■) denote cytokine-mediated proliferative effect; white squares (□), no proliferative effect. T-cell maturation stages as defined in Table 1.

IL-7 and IL-4 are the γ_c-signaling cytokines that induce stronger proliferative responses in T-ALL cells

We next analyzed the magnitude of the T-ALL proliferative responses to the γ_c-signaling cytokines (Figure 2). We observed that IL-7 was not only the cytokine that stimulated a higher percentage of patients but, strikingly, was also inducing a stronger proliferative effect in most of the responders. As shown in Figure 2B, 6 of 18 patients (33%) had a proliferation index greater than 10 in response to IL-7, a considerably higher percentage than for any other cytokine studied (IL-2 0%; IL-4 13%; IL-9 10%; IL-15 17%). In 13 out of 16 patients (81%) that responded to more than one cytokine, including the 3 patients that responded to all cytokines (T-ALL# 2, 5 and 15), IL-7 induced the highest proliferation index (Figure 2A,B). Interestingly, IL-4 was the cytokine that mediated the strongest proliferative effect in 2 of the remaining 3 patients (T-ALL# 6 and 20) that proliferated to more than one cytokine. This is noteworthy since IL-4 was the cytokine that had the second highest effect regarding the number of proliferative patients (Table 2).

We then compared the average proliferative effect induced by each cytokine. As shown in Figure 2C, the mean proliferation index induced by IL-7 was 12.1 ± 4.0 (mean ± SEM), significantly higher than with IL-4 (4.3±1.4; P=0.018; Wilcoxon test), IL-9 (2.4 ± 0.7; P<0.001), IL-2 (1.8 ± 0.2; P<0.001) or IL-15(1.8 ± 0.5; P<0.001). Likewise, the responsiveness to IL-4 was significantly higher than to IL-9 (P=0.018), IL-2 (P=0.011) or IL-15 (P<0.001). The proliferative effects of IL-2, IL-9 and IL-15 were not statistically different (P>0.05). In summary, our studies clearly demonstrate that IL-4 and especially IL-7 were the cytokines that mediated the most intense and more frequent T-ALL proliferative responses.

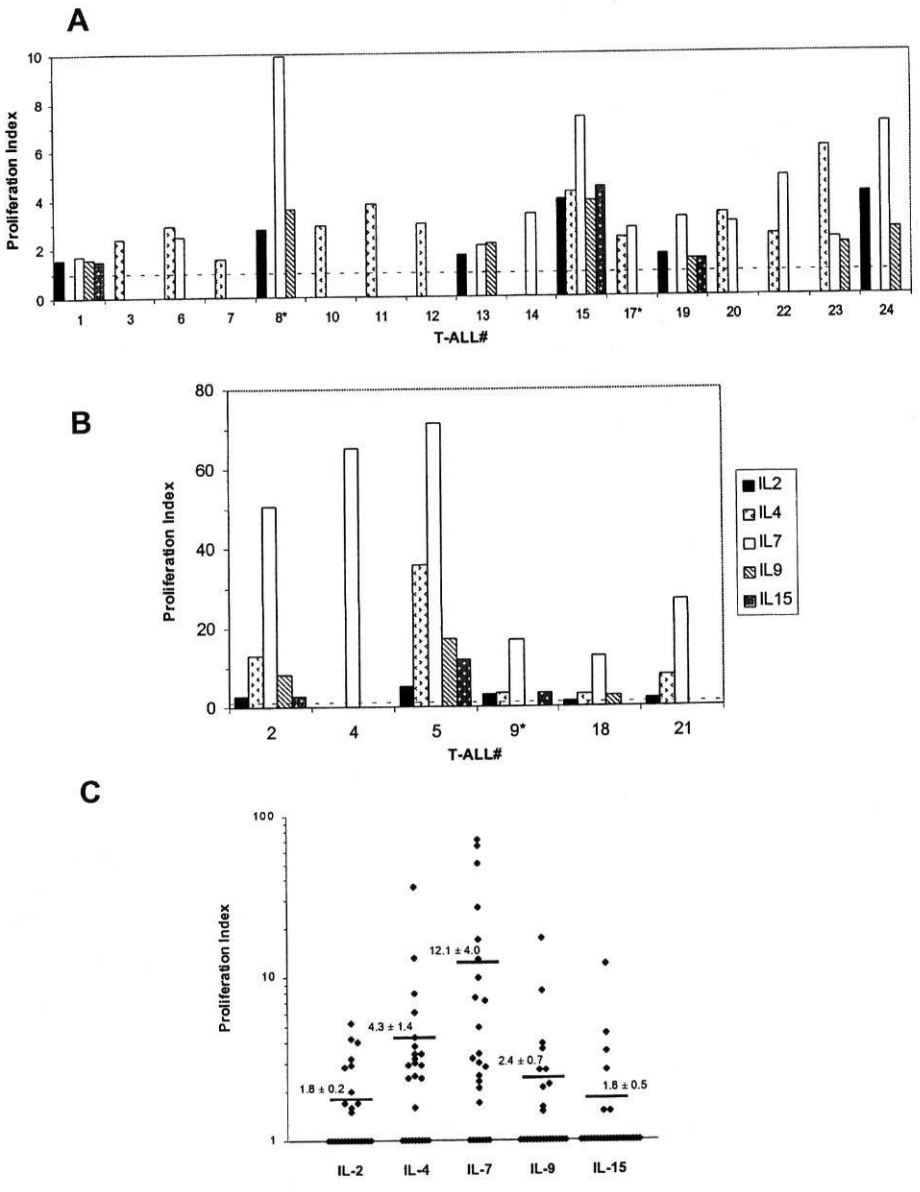

Figure 2. Intensity of the proliferative response of primary T-ALL samples to stimulation with γ_c-signaling cytokines. (A,B) Proliferation index was calculated at 72 hours of culture, except for patients denoted by an asterisk (96 hours). Columns represent only the cytokines that mediated proliferation (PI>=1.5) for each patient. The remaining, unresponsive, cytokines had a PI=1, indicated by the dashed line. No statistically significant inhibitory effects were observed. **(A)** Samples for which the highest proliferative cytokine induced a PI equal to or less than 10. **(B)** Samples for which the highest proliferative cytokine induced a PI of more than 10. **(C)** Average proliferative effect of each cytokine. Each point corresponds to the proliferation index of one T-ALL sample in response to the indicated cytokine. Note that the scale is logarithmic, points in the x-axis correspond to non-proliferative samples (PI=1). Mean PI ± standard error of mean are indicated.

The proliferative response mediated by IL-7 is less transient than that mediated by IL-4 in T-ALL cells

Since IL-7 and IL-4 were the γ_c-signaling cytokines that elicited stronger proliferative responses, we evaluated the kinetics of the T-ALL responses to these cytokines. As shown in Figure 3, the response to IL-7 increased over time in most patients tested (9 of 12 cases; 75%), whereas the effect of IL-4 generally decreased from 72 to 96 hours (60% of the cases).

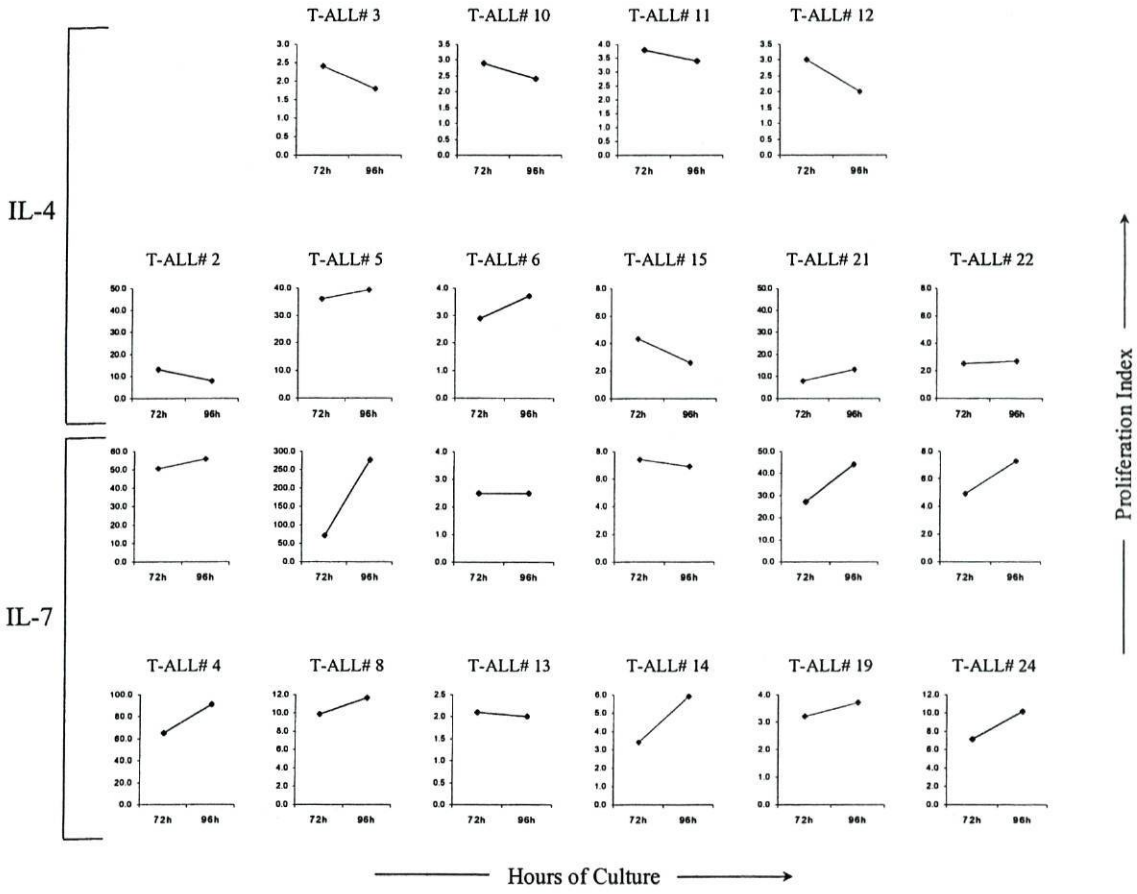

Figure 3. T-ALL cells have differential kinetics of proliferation between 72 and 96 hours of culture in response to IL-4 and IL-7. Proliferation indexes in response to IL-4 and/or IL-7 at 72 and 96 hours were calculated and are indicated for each responsive patient. Note that the proliferative responses were highly variable amongst different patients and hence the scale differs between the plots. Top row concerns to samples that responded solely to IL-4, whereas bottom row concerns to samples that were only IL-7-responsive. The two middle rows correspond to patients that responded to both IL-4 (top) and IL-7 (bottom).

Interestingly, when we compared the patients that responded to both IL-4 and IL-7 (Figure 3, middle rows) we observed that the 3 of the 4 cases in which the proliferative response to IL-4 increased at 96 hours, showed a considerably higher increase with IL-7 (T-ALL# 5, 21 and 22). These results clearly suggest a differential capacity of IL-4 and IL-7 to maintain the proliferative response of primary T-ALL cells over time, and characterize the IL-7-mediated proliferative effect as stronger and more prolonged. Furthermore, the extended effect of IL-7 suggests that this cytokine may play a role in supporting T-ALL clonal expansion. Accordingly, these observations served as the basis for the development of an IL-7-dependent cell line (TAIL7) from the peripheral blood of a patient with T-ALL (Chapter 4).

Responsiveness to γ_c-signaling cytokines in relation to the stage of maturation of T-ALL cells

We next investigated whether there was a correlation between the responsiveness to γ_c-signaling cytokines and the stage of maturation of the malignant T-cells. Since our patient population did not include any stage I samples (pro-T-ALL), we could not evaluate this group of relatively rare and highly primitive T-ALL cells. None of the γ_c-signaling cytokines had an exclusive effect upon any particular maturational stage, with all cytokines inducing proliferation of at least one sample from each T-ALL stage, both using Uckun and EGIL criteria (Table 2). This analysis suggests that γ_c-signaling cytokines have the ability to stimulate T-ALL cells independently of their maturation stage. Subsequently, we evaluated whether the intensity of the proliferative responses was also independent of the maturation stage. No statistically significant differences were found using the Uckun classification, although IL-4 revealed a clear tendency to induce stronger proliferative responses in stage III samples (mature T-ALL) (with a borderline significance, P=0.053; Mann-Whitney Test). This association was confirmed using the EGIL classification. As shown in Figure 4A, the proliferation to IL-4 consistently increased from the most immature, stage II, pre-T-ALL samples to the most differentiated, stage IV, mature T-ALL samples (P=0.035; Kruskal-Wallis Test).

Interestingly, we observed that IL-9 promoted significantly more robust proliferative responses in both pre- and mature T-ALL samples than in cortical T-ALL

samples (P=0.026; Kruskal-Wallis Test) (Figure 4B). In agreement, responsiveness to IL-9 was inversely associated with CD1a expression (P=0.007; Mann-Whitney Test), and was not observed for other cytokines. No significant associations were observed for IL-2, IL-7 or IL-15 (P=0.273, 0.993 and 0.715, respectively).

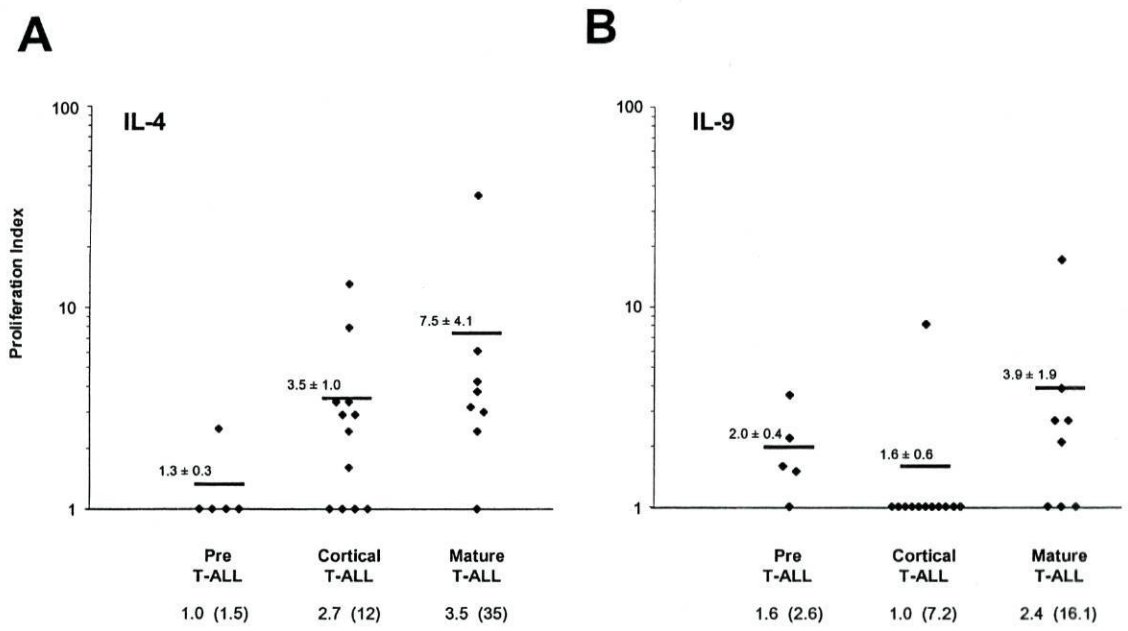

Figure 4. The intensity of the proliferative response to IL-4 and IL-9 depends on the maturation stage of the primary T-ALL samples. Each point corresponds to the proliferation index at 72 hours of one T-ALL sample in response to IL-4 **(A)** or IL-9 **(B)**. Note that the scale is logarithmic, points in the x-axis correspond to non-proliferative samples (PI=1). Samples were grouped according to their maturation stage, as defined by the EGIL criteria [21]. Mean PI ± standard error of mean are indicated for each maturation group. Median PI and range (in brackets) are indicated below the respective maturation group. Results are statistically significant (P=0.035 for IL-4; P=0.026 for IL-9; Kruskal-Wallis Test).

γ_c-signaling cytokines have synergistic effects on T-ALL cell proliferation

IL-7 is present in the normal thymus and bone marrow (produced by stromal cells), as likely are IL-15 (stromal cells), IL-2 and IL-9 (T-lineage cells). Thus, the microenvironment where the leukemia arises produces and is likely modulated by several γ_c-signaling cytokines. Therefore, we analyzed the proliferative responses of T-ALL cells, at 72 hours and/or 96 hours, to all possible combinations of two γ_c-signaling

cytokines. Two cytokines were considered to have a synergistic effect when the proliferation mediated by their combination was higher than the sum of the proliferation mediated independently by each cytokine. An additive effect was scored when the combination-induced proliferation was lower than or equal to the sum of the proliferation of each cytokine, but higher than the proliferative response induced by the respective cytokines alone. As shown in Table 3, all combinations of γ_c-cytokines could be synergistic and/or have an additive effect on T-ALL proliferation, although the strength of the synergistic effect and the percentage of samples involved varied significantly between the various cytokine combinations.

Table 3. Additive and synergistic effects between γ_c-signaling cytokines on proliferation of primary T-ALL cells

COMBINATION EFFECT:		ADDITIVE	SYNERGISTIC	
Cytokine combination		% samples +	% samples +	% synergism *
IL-2	IL-4	14.3 (2/14)	7.1 (1/14)	86
IL-2	IL-7	7.1 (1/14)	28.6 (4/14)	29
IL-2	IL-9	14.3 (2/14)	28.6 (4/14)	17
IL-2	IL-15	14.3 (2/14)	0.0 (0/14)	0
IL-4	IL-7	6.7 (1/15)	6.7 (1/15)	108
IL-4	IL-9	20.0 (3/15)	13.3 (2/15)	94
IL-4	IL-15	6.7 (1/15)	6.7 (1/15)	150
IL-7	IL-9	14.3 (2/14)	21.4 (3/14)	24
IL-7	IL-15	14.3 (2/14)	14.3 (2/14)	66
IL-9	IL-15	7.1 (1/14)	14.3 (2/14)	17

Proliferation of primary T-ALL cells was assessed at 72 and/or 96 hours of culture and the proliferation index (PI) was calculated. The PI of the combination of cytokines was compared to the sum of the PIs induced by each cytokine alone. + Percentage of patients in which an additive or synergistic was observed (the actual number of patients is indicated in brackets) * Average percent increase in PI when comparing the combination of cytokines to the sum of the two cytokines alone – higher values denote stronger synergistic effects

Additive effect: PI (cytokine 1+ cytokine 2)< or = PI (cytokine 1) + PI (cytokine 2) but PI (cytokine 1+ cytokine 2)> PI (cytokine 1) AND PI (cytokine 2). Synergistic effect: PI (cytokine 1+ cytokine 2)> PI (cytokine 1) + PI (cytokine 2)

The most effective cytokine combinations were IL-2 plus IL-9 (synergism/addition in 43% of the samples) and IL-2 plus IL-7 (36%), with the former showing a higher percentage of synergistic cases. In contrast, the average potency of the synergistic effect was higher for IL-2 plus IL-7 (29% increase over the sum of isolated cytokines) than for IL-2 plus IL-9 (17%). Interestingly, despite IL-7 by itself generally inducing the stronger proliferative responses, cytokine combinations that included IL-7 were frequently synergistic/additive (36% of the cases with IL-2; 36% with IL-9; and 29% with IL-15), thus showing that the robust effect of IL-7 can still be potentiated by other cytokines. Overall, these results indicate that when used in combination the γ_c-signaling cytokines can have synergistic/additive proliferative effects on T-ALL primary cells, a property that may be relevant for the biology of T-ALL.

γ_c-cytokine-mediated proliferation is not dependent on induction of calcium flux in T-ALL cells

Previous studies showed that the magnitude of calcium influx resulting from TCR triggering in CD3/TCR-expressing T-ALL cells was small in comparison to normal thymocytes or peripheral blood T-cells [24]. In contrast, IL-7 did not induce calcium flux in normal human thymocytes or mature T-cells [25,26], similar to what has been shown with IL-2 [26]. Therefore, we analyzed calcium mobilization in T-ALL cells after stimulation with different γ_c-cytokines to observe whether T-ALL cells behaved similarly to normal cells or, as for TCR-mediated stimulation, abnormal signaling could be detected. We observed that IL-7 stimulation of T-ALL cells did not promote calcium flux in any of the cases tested, including patients that showed strong proliferation in response to IL-7 (Table 4). Likewise, no calcium flux was detected using as stimuli IL-4 or any other γ_c-cytokine (Table 4). These observations demonstrate that the proliferation of T-ALL cells promoted by γ_c-signaling cytokines does not rely on activation of calcium-dependent pathways, thus mimicking the effect of some of these cytokines (IL-2, IL-7) on normal T-lineage cells.

Table 4. Effect of γ_c-signaling cytokines on calcium mobilization in T-ALL cells

T-ALL#	Factor	Ca^{2+} flux
2	IL-2	-
	IL-4	-
	IL-7	-
	IL-9	-
	IL-15	-
	Ionomycin	+
9	IL-4	-
	IL-7	-
	Ionomycin	+
11	IL-2	-
	IL-4	-
	Ionomycin	+
19	IL-2	-
	IL-7	-
	Ionomycin	+

T-ALL cells from the indicated patients were stimulated as indicated in "Materials and methods" and calcium mobilization was assessed by flow cytometry. Ionomycin was used as positive control. +, calcium flux upon stimulation; -, no calcium flux upon stimulation.

Activation of Jak3 is essential for IL-7-induced proliferation of T-ALL cells

A critical event in cytokine-triggered signaling and cytokine-induced proliferation is the activation of the Jak/STAT pathway, which is a prototypical cytokine-signaling pathway. As described in Chapter 1, engagement of the Jak/STAT pathway has been well documented in normal T-cell biology and in leukemogenesis. In our signaling experiments on T-ALL, we observed that IL-7 induces the tyrosine-phosphorylation of STAT5a/b as well as STAT1 and STAT 3 (see detailed description in Chapter 5 Supplementary Data).

An essential step for IL-7-mediated STAT phosphorylation is the activation of Jak3, which has been shown to occur following IL-7R complex ligation. In fact, Jak3 activation appears to be one of the most upstream events in IL-7-mediated signaling and, in human T-cells, it is involved in the activation of different targets including PI3K and STATs [27]. To test the impact of Jak3 in cytokine-mediated T-ALL proliferation and as a "proof-of-concept" for the targeted inhibition of leukemic cells, we evaluated the effect of the Jak3 inhibitor WHI-P131 on IL-7-promoted stimulation of primary leukemic T-cells. As shown in Figure 5, WHI-P131 completely abrogated the proliferation of primary T-ALL cells induced by IL-7. This inhibition was also observed for the IL-7-dependent TAIL7 cell line (see Chapter 4). Our results indicate that Jak3-dependent pathways appear to be essential for IL-7-mediated leukemic T-cell proliferation. Moreover, these studies illustrate the principle that specific inhibition of cytokine-mediated signals in T-ALL cells, via the blockade of critical signaling events, may constitute a valuable therapeutic strategy.

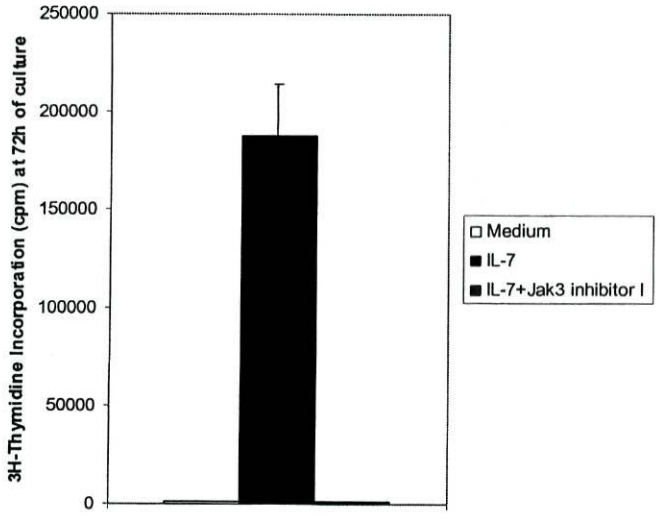

Figure 5. Jak3-specific inhibitor completely abrogates IL-7-mediated primary T-ALL cell proliferation. Primary T-ALL cells were cultured in medium alone, with IL-7 (10 ng/ml) or with IL-7 plus Jak3-specific inhibitor (50 μM) (Jak3 inhibitor I; WHI-P131). Proliferation was assessed after 72 hours of culture. A representative experiment of 1 patient out of 3 tested is shown.

Discussion

A possible regulatory role of γ_c-signaling cytokines in T-ALL pathophysiology is largely unexplored. Because the members of this cytokine subfamily control different aspects of normal T-cell biology in both redundant and critical ways, we examined whether they could also affect T-cell leukemia by assessing their impact on the *ex vivo* proliferation of primary T-ALL cells. We demonstrate that all γ_c-signaling cytokines can induce T-ALL cell proliferation. Our present report confirms the results from previous studies showing that T-ALL blasts have increased proliferation when cultured with IL-7 [2,3,14,28-31], and, in some patients, IL-4 [2,15], as well as IL-2 [2,11-15]. We also demonstrate, for the first time, that IL-9 and IL-15 can likewise function as T-ALL *in vitro* growth factors. IL-2 does not seem to be critical for the generation of mature T-cells from T-cell precursors. In contrast, there is evidence supporting a negative role for IL-2 during thymic development by participating in the elimination of auto-reactive thymocytes [6-8] and preventing the expansion of bipotential T/NK progenitor cells [9]. However, human immature CD4-CD8- double negative thymocytes appear to express IL-2 receptors and proliferate in response to IL-2 [32,33]. Similar results were found in mice, where immature thymocytes have high expression of IL-2R, although the T-cell mitogen ConA is required for clear proliferative responses to IL-2 [34-36]. Thus, IL-2 might have a redundant role in the expansion of early thymocytes and subsequently a non-redundant function during negative selection of double positive thymocytes. However, IL-2 has also been implicated in the negative regulation of bipotential T/NK progenitor cell expansion [9], indicating that the actual role of IL-2 during thymocyte development, if any, is yet to be clearly comprehended. The importance of IL-2 as a growth factor for T-ALL has been suggested not only by the proliferative effect of IL-2 *in vitro* [2,11-15] but also by the existence of IL-2-dependent human T-ALL cell lines, and factor-independent T-ALL cell lines that were initiated by culture with IL-2 [37-39]. Moreover, different studies showed that T-ALL cells can express IL-2Rα, IL-2Rβ [5,10-12], and IL-2Rγ [5]. Interestingly, T-ALL cells appear to be capable of producing IL-2 [13], raising the possibility of the existence of an IL-2 autocrine loop in some T-ALL cases. Our results support the hypothesis that, at least in some patients, IL-2 might be involved in the pathophysiology of T-ALL.

It seems presently clear that IL-4 plays no significant role during thymocyte development, in contrast to its well-established role in mature T-cell differentiation towards the TH2 phenotype (reviewed in Refs. [40,41]). The studies regarding the effect of IL-4 on T-ALL are scarce and conflicting. Two early reports with very few samples showed that IL-4 could induce proliferation in 2 of 2 [2] and 4 of 10 [15] T-ALL samples tested, whereas a recent study indicated that in 7 of 37 cases, IL-4 prevented spontaneous apoptosis of *in vitro* cultured T-ALL cells without mediating cell proliferation [5]. Our results support the former reports by showing that IL-4 induces proliferation in the majority (16 of 25) of the samples studied.

IL-7 was suggested to have the ability to act as an oncogene *in vivo*, since transgenic mice overexpressing IL-7 develop lymphoid malignancies [4]. T-ALL cells express the receptor for IL-7 [2,3,28,42,43], and the anti-apoptotic effect of IL-7 on T-ALL correlates with IL-7Rα surface expression [5]. Moreover, various groups have demonstrated that IL-7 stimulates T-ALL proliferation *in vitro* [2,3,14,28-30]. However, these studies utilized only few samples each. Here, we reconfirmed these results using a more significant number of T-ALL cases and showed that IL-7 is the γ_c-signaling cytokine that induces proliferation in a greater number of samples (18 of 25). Strikingly, we also demonstrated that IL-7 induces the highest average proliferation index, indicating that IL-7 has a strong proliferative capacity over primary T-ALL cells. Accordingly, IL-7-mediated T-ALL cell growth appears to be less transient than the one resulting from culture with IL-4, the γ_c-signaling cytokine that induced the second-most significant proliferative effect. The proliferative effect of IL-7 does not appear to be associated with induction of differentiation of primary leukemic cells. Preliminary studies in three patients (T-ALL# 5, 19 and 22) showed the absence of immunophenotypic changes after culture with IL-7 (data not shown). Despite the essential role of IL-7 for normal T-cell development, our preliminary results suggest that IL-7 is not sufficient to induce maturation of T-ALL cells. In accordance, Touw et al. [2] have reported that the maturation-inducing effects of IL-7 on T-ALL cells were very limited, as evidenced by the failure of IL-7 to upregulate differentiation antigens in leukemic T-cells. Likewise, Dibirdik et al. [3] have shown that IL-7 stimulated the *in vitro* proliferation of T-ALL cells without inducing differentiation: IL-7-cultured colony blasts were immunophenotipically

identical to fresh bone marrow leukemic blasts. Identical results were obtained in another study, regarding the effects of IL-2, IL-4 and IL-7 [44].

Our present study shows for the first time that IL-9 and IL-15 might be implicated in the proliferation of some T-ALL cases. The response of T-ALL to IL-9 was equivalent to that induced by IL-2 in both frequency and magnitude of the proliferation. IL-9 was suggested to be critical for the early stages of human T-cell development [45], and both IL-9 and its receptor are present in human thymocytes, suggesting the possibility of an autocrine regulation of early T-cell development by IL-9. Moreover, IL-9 prevents dexamethasone-induced T-cell apoptosis [46]. A link between IL-9 and T-cell malignancies has also been suggested. IL-9 has a clear *in vitro* anti-apoptotic [16] and proliferative effect [17] in thymic T-cell lymphomas. Additionally, IL-9 transgenic mice have increased incidence of thymic T-cell lymphomas, suggesting that deregulated IL-9 expression could be involved, possibly through an autocrine/paracrine loop, in the development of some T-cell malignancies [47]. Future studies will allow the evaluation of whether a similar phenomenon occurs in T-ALL.

We found that IL-15 mediated proliferation in only 6 of 25 cases studied, with proliferative responses of a magnitude identical to those of IL-2 and IL-9. A possible functional role of IL-15 in T-ALL expansion *in vivo* is arguable. Normal thymocytes do not seem to require IL-15 [48]. However, studies with double knockout mice have demonstrated that IL-15 plays a redundant role with IL-7 in early thymic T-cell development (pro-T1 stage or earlier) [49], and IL-15 mRNA is expressed in the thymic epithelial [9] and bone marrow stromal cells [50]. Moreover, long-term BM stromal cell cultures contain IL-15 protein [50]. Since IL-15 is likely produced in the microenvironments where the leukemia arises and appears to be able to modulate normal thymocytes, it is conceivable that deregulated expression of IL-15 could influence some T-ALL cases. The validation of this hypothesis requires further studies.

A possible association between γ_c-signaling cytokine-induced proliferation and T-ALL maturation stage has not been previously explored. However, a recent study demonstrated that IL-7 prevented spontaneous apoptosis preferentially on T-ALL cells that expressed cortical and mature immunophenotypes [5]. We have not observed this association regarding proliferation to IL-7 and T-ALL maturation stage. Likewise, IL-2

and IL-15 did not preferentially induce proliferation upon any particular stage of maturation. In contrast, responsiveness to IL-4 progressively increased with the acquisition of maturation markers. Interestingly, cortical T-ALL cells, characterized by expression of CD1a, were mostly unresponsive to IL-9. Different studies have shown that CD1a-positive patients present significantly better *in vivo* responses to prednisone and a more favorable outcome than patients with other immunophenotypic T-ALL subsets [51,52]. Based on our studies, it will be interesting to evaluate whether responsiveness to IL-9 may serve as a predictive marker for clinical outcome in T-ALL. Importantly, IL-9 was suggested to be an essential cytokine for human early T-cell development [45], and a recent work has shown that expression of the oncogenic transcription factor LYL1 in T-ALL is associated with increased expression of IL-9R and an immature CD1 negative phenotype [22]. A considerable portion of the IL-9-responsive T-ALL samples in our study had the same immunophenotype, raising the interesting question of whether IL-9 can be an *in vivo* positive regulator of LYL1+ T-ALL cells.

We showed that γ_c-signaling cytokines can have synergistic effects on T-ALL cell proliferation. The pairs of cytokines tested were all synergistic or additive in at least one case, suggesting that the combination of these cytokines can augment the effect of each independent cytokine in some T-ALL cases. Notably, the generally strong proliferative effect of IL-7 could be increased by combination with other γ_c-signaling cytokines. Our results apparently contradict those of Masuda et al. [15] that observed no synergistic effects between IL-7 and IL-2 or IL-4 in three T-ALL patients tested. However, the small number of patients analyzed in that study might explain the apparent discrepancy. Our results suggest that combinations between γ_c-signaling cytokines are synergistic in a relatively small number of cases, and therefore could be easily overlooked in very small scale studies. Careful analysis of another study in which no synergisms were found between IL-7 and IL-2 in two T-ALL samples, revealed that the combination of these two cytokines had an additive effect in both patient samples [2]. Interestingly, IL-7 and IL-2 act synergistically to stimulate the proliferation of immature CD4- CD8- double negative thymocytes [53,54]. Moreover, IL-2 has been shown to act in synergy with Steel factor, the ligand for c-kit receptor, in the T-ALL cell line PER-423 [37], suggesting that IL-2 has the ability to act in concert with other cytokines to regulate T-ALL cell function. In human

early T-cell development, IL-9 has been proposed to complement the effect of IL-7 by non-redundantly contributing to overcome a minimum threshold of activation. Hence, although acting upon common intracellular pathways to IL-7, IL-9 would be essential for optimal T-cell production [45]. Our results seem to be in agreement with the ability of IL-9 to act synergistically with IL-7. Moreover, our results underline the potential relevance of these combinations of related cytokines for *in vivo* expansion of T-ALL.

Many growth factors exert their biologic activity by increasing the concentration of free cytoplasmic calcium [55,56], and TCR-dependent T-cell activation is associated with calcium mobilization [57]. However, this does not appear to be the case for various cytokines [58]. In accordance with a previous report [44], we did not observe any calcium flux mediated by IL-7 or any other γ_c-cytokine in T-ALL cells that proliferated in response to those cytokines, indicating that they trigger T-ALL cell proliferation through calcium-independent pathways. Similarly, IL-7 does not induce calcium flux in normal human thymocytes or mature T-cells [25,26]. The absence of calcium mobilization correlates with the inability of IL-7 to induce phosphorylation of phospholipase C-γ1 (PLC-γ1), both in normal and leukemic T-cells [25,44]. Thus, no abnormal activation of calcium-dependent pathways is apparent in response to γ_c-signaling cytokines in T-ALL primary cells.

Constitutive activation of Jak/STAT pathway has been described in T-ALL and suggested to contribute to the development of the malignancy [59,60]. Our studies demonstrated that Jak3 is required for IL-7-mediated proliferation of primary T-ALL cells. IL-7 induces also tyrosine phosphorylation of STAT1, STAT3 and STAT5, and DNA-binding activity of at least STAT5, in the T-ALL cell line TAIL7 (see Chapter 5 Supplementary Data). Jak/STAT pathway might be relevant for T-ALL expansion *in vivo* even in those cases where constitutive activation is not observed, because T-ALL cells survive and proliferate in a microenvironment that produces factors, such as IL-7, that likely continuously stimulate this pathway. Importantly, our results support the view that specific inhibition of cytokine-mediated signals in T-ALL cells may constitute a valuable therapeutic strategy.

In summary, our present studies demonstrate that IL-2, IL-4, IL-7, IL-9 and IL-15 can generally mediate the *in vitro* proliferation of primary T-ALL cells, and we propose

that some of these cytokines, most notably IL-7, might play an important regulatory role in T-ALL biology.

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Chapter 3

IL-7 PROMOTES SURVIVAL AND CELL CYCLE PROGRESSION OF T-ALL CELLS BY DOWNREGULATING THE CYCLIN-DEPENDENT KINASE INHIBITOR $p27^{kip1}$

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Abstract

In normal T-cell development IL-7 functions as an antiapoptotic factor by regulating Bcl-2 expression in immature thymocytes and mature T-cells. Similar to what occurs in normal immature thymocytes, prevention of spontaneous apoptosis by IL-7 in precursor T-cell acute lymphoblastic leukemia (T-ALL) cells correlates with upregulation of Bcl-2. IL-7 is also implicated in leukemogenesis since IL-7 transgenic mice develop lymphoid malignancies, suggesting that IL-7 may regulate the generation and expansion of malignant cells. Here we show that in the presence of IL-7, T-ALL cells not only upregulated Bcl-2 expression and escaped apoptosis but also progressed in the cell cycle resulting in sequential induction of cyclin D2 and cyclin A. Downregulation of $p27^{kip1}$ was mandatory for IL-7-mediated cell cycle progression and temporally coincided with activation of cyclin dependent kinase (cdk)4 and cdk2 and hyperphosphorylation of Rb. Strikingly, forced expression of $p27^{kip1}$ in T-ALL cells not only prevented cell cycle progression but also reversed IL-7-mediated upregulation of Bcl-2 and promotion of viability. These results show for the first time that a causative link between IL-7-mediated proliferation and $p27^{kip1}$ downregulation exists in malignant T-cells. Moreover, these results suggest that $p27^{kip1}$ may function as a tumor suppressor gene not only because it is a negative regulator of cell cycle progression but also because it is associated with induction of apoptosis of primary malignant cells.

Introduction

In normal T-cell development IL-7 plays a nonredundant role as an antiapoptotic factor by regulating Bcl-2 expression in immature thymocytes and mature T-cells [1-4]. Mice deficient in IL-7 receptor are lymphopenic due to a defect in cell expansion at an early stage of differentiation and the few mature T-cells that develop are functionally impaired [2]. Both defects are completely rescued by overexpression of the anti-apoptotic bcl-2 gene [3,4]. Similarly, mice deficient in IL-7 exhibit an early defect in lymphopoiesis and developmental transition of their immature thymocytes to a T-cell committed fate is accompanied by a striking loss of Bcl-2 protein expression and an increased proportion of cells in G0/G1 stage of the cell cycle [5,6]. Culture of immature thymocytes from IL-7 deficient mice with IL-7, caused upregulation of Bcl-2 protein and cell survival, indicating that during T-cell lineage developmental transition and prior to T-cell antigen receptor rearrangement, IL-7 mediated signals are linked to an anti-apoptosis mechanism and cell cycle progression [6].

T-cell acute lymphoblastic leukemia (T-ALL) results from clonal expansion of hemopoietic progenitors that have undergone malignant transformation at distinct stages of differentiation and they may retain certain features of their normal counterparts. Indeed, earlier studies determined that similar to normal immature thymocytes, leukemic blasts from a number of T-ALL patients express functional IL-7 receptors [7]. Subsequently it was reported that IL-7 was stimulatory on blast colony formation and DNA synthesis, suggesting that IL-7 may play an important regulatory role in the biology of T-ALL [7,8]. More recently it was determined that IL-7 prevents spontaneous apoptosis of pre-T-ALL cells and this effect correlates with upregulation of Bcl-2 [9]. IL-7 is produced in the bone marrow and the thymic stroma and thus, it is present in the microenvironments in which the malignant T-cells develop. Importantly, IL-7 has also been implicated in leukemogenesis since IL-7 transgenic mice develop lymphoid malignancies [1]. Therefore, the question arises whether IL-7 is involved not only in promotion of viability but also in clonal expansion of T-ALL cells.

In the present study we show that during culture with IL-7, primary T-ALL cells not only escaped apoptosis but also progressed to the S+G2/M phase of the cell cycle.

IL-7 induced sequential expression of cyclin D2 and cyclin A, and dramatically increased the enzymatic activity of cyclin dependent kinases (cdk) cdk4, cdk6 and cdk2. This event temporally correlated with hyperphosphorylation of Rb and progression to the S phase of the cell cycle. Amongst the CIP/KIP and INK4 family cdk inhibitors, only $p27^{kip1}$ was dramatically decreased at the time of cdk2 activation and S phase progression. Downregulation of $p27^{kip1}$ had a causative role in IL-7 mediated cell cycle progression, because forced sustained expression of $p27^{kip1}$ prevented activation of cdk2, hyperphosphorylation of Rb and cell cycle progression. Importantly, viability studies revealed that forced sustained expression of $p27^{kip1}$ during culture with IL-7 not only prevented cell cycle progression but also prevented IL-7-mediated Bcl-2 expression and reversed the effect of IL-7 on the promotion of viability. Thus our results show that IL-7 mediates both Bcl-2 expression, leading to prevention of apoptosis, and cell cycle progression by downregulating $p27^{kip1}$ cdk inhibitor in primary T–ALL cells.

Materials and Methods

Primary T-ALL samples and immunophenotypic analysis. T-ALL cells were obtained from the peripheral blood and/or the bone marrow of patients with high leukemia involvement (85-100%). Informed consent and Institutional Review Board approval was obtained for all sample collections. Samples were enriched by density centrifugation over Ficoll-Hypaque and then washed twice in RPMI-1640 supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 2 mM L-glutamine (further referred to as RPMI-10). Expression of cell surface molecules was determined by direct labeling using standard methodology [10]. Fc receptors were blocked by incubation with mouse Ig before the addition of the specific mAbs. The mAbs used were fluorescein isothiocyanate (FITC)-conjugated anti-CD3 and CD2 and phycoerythrin (PE)-conjugated anti-CD4, CD5, CD7 and CD8, CD19, CD13 and CD33. Irrelevant isotype-matched antibodies were used as negative controls. Samples were analyzed in a Coulter Elite or XL flow cytometer, and data were acquired in listmode files. At least 5,000 positive events were measured for each sample.

***In vitro* culture.** Cells isolated by density centrifugation over Ficoll-Hypaque were cultured in 24-well plates as $2x10^6$ cells/ml at 37°C with 5% CO2 in RPMI-10 without any cytokine (medium alone) or with 10 ng/ml IL-7 (Endogen, Woburn, MA). At the indicated time points, cells were harvested and processed as indicated below for assessment of viability, cell cycle progression and preparation of lysates for Western blotting and immunoprecipitation.

Proliferation assays. Cells were cultured in flat-bottom 96-well plates as $2x10^6$ cells/ml at 37°C with 5% CO2 in RPMI-10 without any cytokine or with 10 ng/ml IL-7 for the indicated time points. Cells were incubated with ^{3}H-Thymidine (1 μCi/well) for 16 hours prior to harvest. DNA synthesis, as measured by ^{3}H-Thymidine incorporation, was assessed using a liquid scintillation counter. Proliferation index was calculated as thymidine incorporation of IL-7 cultured cells over thymidine incorporation of medium alone cultured cells.

Assessment of cell viability. Quantitative determination of viability of the malignant cells after culture under different conditions was performed by using an Annexin V-based apoptosis detection kit and the manufacturer's protocol (R&D Systems, Flanders, NJ). Briefly, cells were suspended in the appropriate binding buffer, stained with FITC-conjugated Annexin V and propidium iodide at room temperature for 15 minutes, and subsequently analyzed by flow cytometry.

Cell cycle analysis. Determination of the percentage of cells at each stage of the cell cycle was performed by assessment of DNA content after staining with propidium iodide as described before [11]. Briefly, 5 x 10^5 cells per sample were resuspended in 0.5 ml PBS and then fixed with ice-cold 80% ethanol. Propidium iodide was added at a final concentration of 2.5 μg/ml, ribonuclease A was added at 50 μg/ml, and samples were incubated for 30 min at 37°C in the dark. Flow cytometric analysis was performed using Lysis II (Becton Dickinson, Mountain View, CA) and/or XL2 software, and analysis of

cell cycle histograms was carried out using ModFit LT (Verity, Topsham, ME) or WinCycle DNA Analysis software (Phoenix Flow Systems, San Diego, CA).

Determination of malignant origin of IL-7-responsive cells. To determine the clonality of IL-7-responsive cells two approaches were undertaken. In the first approach, cells from patients for which patient-specific T-cell receptor (TCR) rearrangements were identified were cultured with IL-7. After 72 hours of culture S+G2/M cells were sorted by flow-activated cell sorting (FACS) using Hoechst 33342 dye (Sigma-Aldrich, St. Louis, MO) and the presence of the specific rearrangement was examined by RT-PCR as previously described [12]. PCR products were electrophoresed through 2% agarose gels containing ethidium bromide and visualized under UV light. Normal T-cells and cells from other T-ALL patients were used as negative controls. In the second approach, for patients without specific rearrangements but with low or negative CD3 expression, cells were cultured with IL-7. After 72h of culture, absence of CD3 expression of cells in S+G2/M was assessed by flow cytometry, using propidium iodide and FITC-conjugated CD3 (Pharmingen, San Diego, CA).

Intracellular staining. Expression of Bcl-2 protein family members, Bcl-2, Bcl-x_L, Bax and Bad was assessed at 72 hours of culture by intracellular staining. Cells were fixed in 0.1 % formaldehyde for 30 minutes at 4°C, washed in PBS and resuspended in 1x Perm/Wash Solution (Pharmingen, San Diego, CA). The antibodies used were mouse monoclonal FITC-conjugated anti-Bcl-2 (Dako, Glostrup, Denmark) and Bad (Transduction Laboratories, Lexington, KY), and rabbit polyclonal purified anti-Bcl-x_L (Santa Cruz Biotechnology, Santa Cruz, CA) and Bax (Transduction Laboratories, Lexington, KY). FITC-conjugated goat anti-rabbit (Southern Biotechnology Associates, Birmingham, AL) was used as secondary antibody. Irrelevant isotype-matched antibodies were used as negative controls. Propidium iodide was added to each sample in order to distinguish live from apoptotic cells. Samples were analyzed by flow cytometry (XL2 software; Beckman-Coulter, Fullerton, CA). Results were expressed as the ratio of mean intensity of fluorescence (MIF) of the specific antibody stain over the MIF of negative control antibody.

Immunoblotting, immunoprecipitation, and *in vitro* kinase reactions. Following the indicated conditions and time intervals of culture, cell lysates were prepared, and equal amounts of protein (50 μg/sample) were analyzed by 10% sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes, and immunoblotted with the indicated mAbs or antiserum. Cyclin A, cyclin D2, cyclin E, cdk2, cdk4, cdk6, and $p16^{INK4a}$ antiserum were purchased from Santa Cruz Biotechnology, $p21^{cip1}$ mAb from Upstate Biotechnology (Lake placid, NY), and $p27^{kip1}$ mAb from Transduction Laboratories. To examine the phosphorylation status of Rb, proteins were analyzed by 6% SDS-PAGE, transferred onto nitrocellulose membrane, and blotted with Rb-specific mAb (PharMingen). After immunoblotting with mAbs or antiserum, immunodetection was performed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (1:5,000) or anti-rabbit IgG (1:10,000) (Promega, Madison, WI) as indicated by the host origin of the primary antibody and developed by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Stripping and reprobing of the immunoblots were done as described [13].

For *in vitro* kinase reactions, immunoprecipitations were done using equal amounts of protein (200 μg/sample) with anti-cdk2-specific antiserum agarose conjugate (Santa Cruz Biotechnology). *In vitro* kinase reactions were performed using histone H1 (Sigma) as exogenous substrate, according to described protocol [14]. After immunoprecipitation with anti-cdk4-specific antiserum, *in vitro* kinase reactions were performed by using Rb-glutathione-*S*-transferase (Santa Cruz Biotechnology) as exogenous substrate as described [14]. Reactions were analyzed by 10% SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membrane, and exposed to x-ray film.

Rapamycin and VP22/$p27^{kip1}$ fusion protein. Rapamycin was purchased from Sigma-Aldrich (St. Louis, MI) and was added in the culture at a final concentration of 100 nM. VP22 fusion proteins allow expression of recombinant proteins in cells without directly transfecting them [15]. Forced expression of exogenous $p27^{kip1}$ was achieved by using a VP22/$p27^{kip1}$ fusion protein produced in COS cells as previously described [16]. Because VP22/$p27^{kip1}$ fusion protein is tagged with 6x histidine, it allows preparation of purified

fusion protein from cell lysates of COS transfectants by the use of nickel-chelated agarose columns (Pierce, Rockford, IL). Cells were cultured as described above with or without IL-7 plus VP22/$p27^{kip1}$ or VP22 control (1:10 vol/vol) and/or 100 nM rapamycin.

Bcl-2 antisense oligonucleotides. Bcl-2 antisense and scrambled control oligonucleotides (Biomol Research Laboratories, Plymouth Meeting, PA) were added to culture at 100 nM as a complex with Lipofectin (Life Technologies [Gibco], Gaithersburg, MD), according to a method adapted from the manufacturer's protocol. Briefly, cells were washed twice in AIM V serum-free medium, cultured in 24-well plates and immediately spun down. Oligonucleotides were resuspended in sterile double distilled H_2O and incubated for 15 minutes with Lipofectin and AIM V medium at room temperature, before adding to the cell culture. As an additional control sterile ddH_2O was used without any oligonucleotide. Cells with oligo mix were incubated at 37 C for 4 hours. The cells were harvested, washed and recultured in RPMI-10 with the same concentration of oligonucleotide mix as previously used. Bcl-2 protein expression, viability and proliferation were assessed at 72 hours of culture.

Results

IL-7 promotes viability of T-ALL cells by upregulating Bcl-2 protein expression.

Highly enriched leukemic cells were isolated from the bone marrow or peripheral blood of pediatric T-ALL cases. Of the 21 cases analyzed, all were positive for the pan-T-cell marker CD7 as well as for CD2 and/or CD5. Eight cases were CD3 negative and the remaining had a more mature phenotype and expressed this marker on the surface. Based in these findings, patients were classified according to the maturation stage of the malignant T-cells [17] (Table 1). No expression of the B-lineage differentiation antigen CD19, or the myeloid lineage markers CD13 and CD33 was detected (data not shown).

Table 1. Immunophenotype, classification and response to IL-7 of 21 patients with T-ALL

Patients	CD2	CD5	CD7	CD3	CD4	CD8	Stage	Viability response to IL-7	Proliferative response to IL-7
1	+	+	+	+	−	−	III	ND	+
2	+	+	+	+	+	+	III	−	−
3	+	+	+	−	+	+	II	+	+
4	+	+	+	+	−	−	III	+	+
5	+	+	+	+	−	−	III	+	+
6	−	ND	+	+	+	−	III	ND	−
7	+	+	+	−	+	−	II	+	+
8	+	+	+	−	+	+	II	ND	+
9	+	+	+	+	ND	+	III	−	−
10	+	+	+	+	−	+	III	+	−
11	+	+	+	+	−	+	III	+	−
12	ND	+	+	−	ND	−	II	ND	+
13	+	+	+	+	−	−	III	+	+
14	−	+	+	+	+	−	III	+	+
15	−	+	+	−	+	−	II	ND	−
16	+	+	+	−	−	+	II	ND	+
17	+	+	+	−	+	−	II	+	+
18	+	+	+	+	+	+	III	ND	+
19	+	+	+	−	+	+	II	+	+
20	+	+	+	+	+	−	III	ND	+
21	+	+	+	+	−	+	III	ND	−

T-cell maturation stages were defined as described [17]: Stage I (Pro-TL): $CD7^+$ $CD2^-$ $CD5^-$ $CD3^-$. Stage II (Immature TL): $CD7^+$ (CD2 and/or CD5)$^+$ $CD3^-$. Stage III (mature TL): $CD7^+$ $CD2^+$ $CD5^+$ $CD3^+$. IL-7 indicates interleukin-7; T-ALL, T-cell acute lymphoblastic leukemia; ND, not determined.

Responsiveness of leukemic cells to IL-7 was determined by inhibition of spontaneous apoptosis during *in vitro* culture. Ten out of twelve patients studied, had a significant response to IL-7 as determined by increased viability assessed by annexin V and propidium iodide staining followed by FACS analysis, as shown in Figure 1. The effect of IL-7 in promotion of viability was obvious at 24 hours of culture and maximal difference in viability between media and IL-7 cultures was observed at 72 hours. Dose-response curves in responding samples indicated that concentration of 10 ng/ml provided the saturating amount of IL-7 required to induce maximal response. Therefore, all subsequent experiments were performed at this time interval, using the optimal concentration of IL-7.

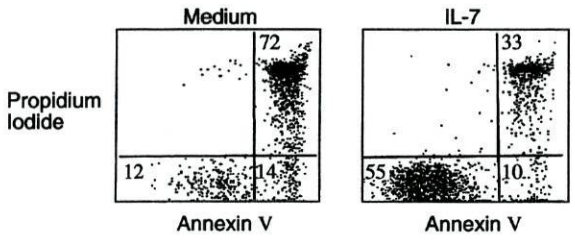

Figure 1. IL-7 prevents apoptosis of T-ALL cells during in vitro culture. T-ALL cells were cultured either in medium alone or in the presence of IL-7. After 72 hours of culture, cells were harvested, stained with annexin V and propidium iodide, and viability was determined by FACS analysis. Results obtained from one representative patient are shown and were similar to those observed in 10 of 12 patients studied.

Analysis of Bcl-2, Bcl-x_L, Bad and Bax protein expression by flow cytometry revealed that all proteins were detected in primary T-ALL cells (data not shown). Culture with IL-7 increased Bcl-2 expression as determined by the increase in mean intensity of fluorescence (MIF) compared to culture with media alone (Table 2), a finding consistent with previous results [9]. In contrast, no changes in Bcl-x_L, Bad or Bax expression correlated with culture in IL-7 and IL-7-mediated promotion of viability of T-ALL (data not shown). To determine whether upregulation of Bcl-2 was simply a correlative event or it had a causative role in IL-7-mediated promotion of viability of T-ALL cells, Bcl-2 antisense oligonucleotides were added during culture of T-ALL cells with IL-7. As shown in Table 2, co-culture with Bcl-2 antisense oligonucleotides prevented IL-7 mediated Bcl-2 protein upregulation and reversed IL-7-mediated in-vitro survival of T-ALL cells. These results indicate that upregulated Bcl-2 had a critical functional significance for the IL-7-mediated promotion of viability of T-ALL cells.

Table 2. Effect of bcl-2 anti-sense oligonucleotides on Bcl-2 protein expression and IL-7-mediated promotion of viability in T-ALL cells

	Percentage of live cells	Bcl-2 protein expression (MIF)
Media	**34.21**	**6.96**
IL-7	**48.64**	**12.25**
IL-7 + Control oligonucleotide	**51.83**	**14.26**
IL-7 + bcl-2 antisense	**34.08**	**5.01**

T-ALL cells were cultured with media, IL-7, or IL-7 and bcl-2 antisense or control oligonucleotides. Bcl-2 protein expression and percentage of viable cells were examined in 6 patients, and results of one representative experiment are shown. The effect of bcl-2 anti-sense oligonucleotide was examined in two patients and results of one experiment are shown. MIF, mean intensity of fluorescence.

IL-7 promotes cell cycle progression of T-ALL cells

As mentioned above, besides promotion of viability, IL-7/IL-7 receptor mediated signals have a critical role in cell expansion at early stages of T-cell differentiation [5,6,18]. To determine whether, besides increased survival, IL-7 might mediate clonal expansion of primary T-ALL cells, we examined DNA synthesis following culture of leukemic cells with either media or with IL-7. As shown in Figure 2A for one representative case, in 14 out of 21 tested patients, addition of IL-7 resulted in increased DNA synthesis compared to medium alone, as determined by assessment of ^{3}H-thymidine incorporation at various time intervals of culture. The mean value of the proliferation index among these 14 patients was 17.3 and the median value was 8.5 (range 2.8-78.8). In contrast to the response of primary T-ALL cells to IL-7, no proliferation was observed when primary peripheral blood T lymphocytes from healthy volunteer donors were examined (data not shown). To examine whether increased ^{3}H-thymidine incorporation in IL-7 cultures was only due to the higher percentage of viable T-ALL cells or, alternatively, represented cell expansion, cell cycle analysis was performed. Culture with IL-7 resulted in increase of cells in the S and G2/M phases of the cell cycle (Figure 2B) demonstrating that IL-7 not only prevented apoptosis but also induced proliferation of primary T-ALL cells.

To confirm that proliferating cells in the *in vitro* cultures were of malignant origin and not normal T-cells contaminating the sample, two approaches were undertaken: First, cycling cells at the S+G2/M phases of the cell cycle were isolated by cell sorting and examined by PCR using patient-specific primers for the detection of a specific clonal TCR rearrangement identified at diagnosis. Such specific rearrangements were identified in the cycling cells confirming that these cells were of malignant origin and were absent in cells from other patients or from normal donors (Figure 2C). Second, for patients without specific rearrangements but with low or negative CD3 expression, cells at the S+G2/M phases were sorted after 72 hours of IL-7 culture and absence of CD3 expression was assessed by flow cytometry, using propidium iodide and FITC-conjugated anti-CD3. This immunophenotypic finding of the cells undergoing cell cycle progression during culture with IL-7 was identical to that of fresh leukemic cells at the time of diagnosis (data not shown). Therefore, both approaches determined that the proliferating cells during *in vitro* culture with IL-7 were of T-ALL origin.

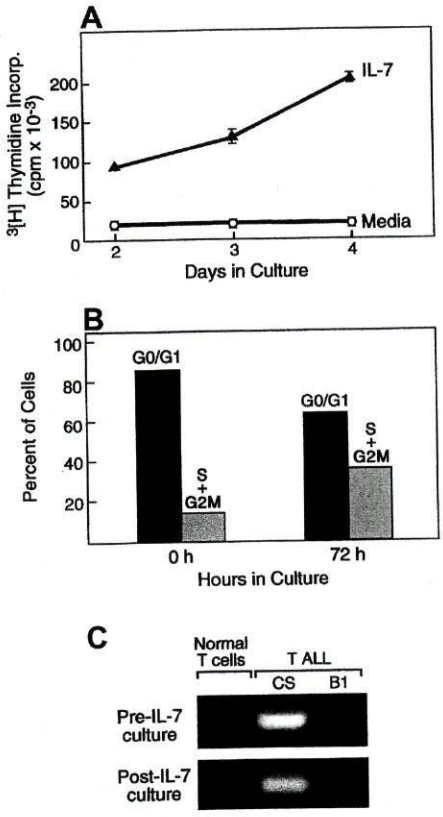

Figure 2. IL-7 promotes cell cycle progression in T-ALL cells. (A) T-ALL cells were cultured for 72 hrs in media alone or in the presence of IL-7 and proliferation was examined by thymidine incorporation. **(B)** At the same time interval IL-7 cultured cells were isolated and percentage of cells at each phase of the cell cycle was examined as described in Materials and Methods. **(C)** Cells cycling in response to IL-7 are of malignant origin. After culture with IL-7, cells at the S+G2/M phases of the cell cycle were isolated by cell sorting and examined by RT-PCR using patient-specific primers for the detection of a specific clonal TCR rearrangement identified at diagnosis. Results from one representative patient (P4) among three patients studied are shown. Primers used were specific for patient P4 and generated a PCR product from P4 (CS) but not from patient P2 (B1) or from normal T cells.

IL-7 mediates upregulation of cyclins and activation of cyclin-dependent kinases in T-ALL cells.

The transition from the G0/G1 to the S phase of the cell cycle is affected by exogenous factors and is positively regulated by cyclin/cyclin dependent kinase (cdk) complexes, which phosphorylate various intracellular substrates including Rb [19]. Inactivation of Rb due to its phosphorylation results in release of E2F transcription factors thereby allowing them to initiate DNA binding and transcription of S phase genes [20-22]. To determine the molecular mechanism(s), by which IL-7 mediated cell cycle progression of T-ALL cells, we examined the expression of cyclins, expression and activation of cdks and expression of cdk inhibitors. IL-7 led to upregulation of cyclin D2 and cyclin A in a sequential manner. Cyclin D2, which is an early G1 cyclin peaked at 24 hours of culture, whereas cyclin A, which is an S-phase cyclin, was maximal at later time points of culture (Figure 3A).

IL-7 slightly increased expression of cdk2 and cdk4 and had no effect on expression of cdk6 (Figure 3B). Importantly, in contrast to the minimal effect on the expression of these cdks, IL-7 induced a striking activation of cdk4 (data not shown) and cdk2 (Figure 3C) as determined by *in vitro* kinase reaction. To determine the *in vivo* significance of these findings we examined the phosphorylation status of endogenous Rb, which is one of the most critical substrates of the enzymatic activity of cdks *in vivo*. Culture with IL-7 resulted in hyperphosphorylation of Rb (Figure 3D), indicating that IL-7-induced cdk activation occurred *in vivo*.

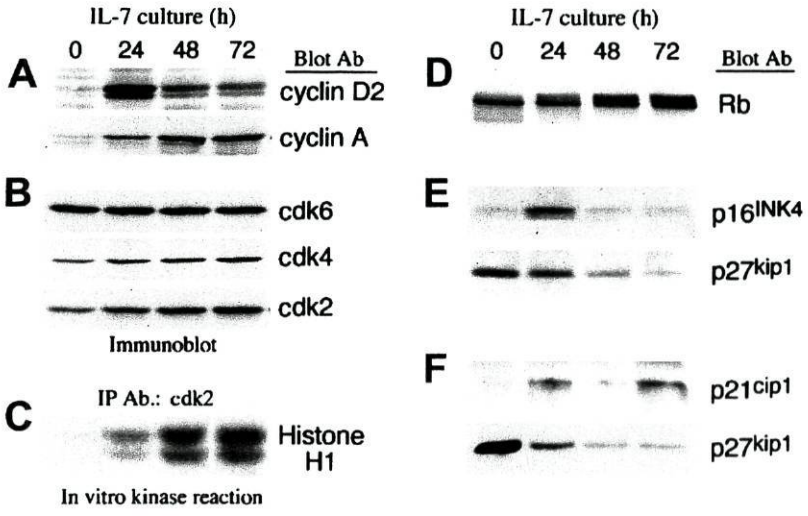

Figure 3. IL-7 mediates upregulation of cyclins and activates cyclin-dependent kinases due to downregulation of cyclin dependent kinase inhibitor $p27^{kip1}$ in T-ALL cells. (A,B) T-ALL cells were cultured with IL-7 for the indicated time intervals, lysates were analyzed by SDS-PAGE and immunoblotted with an antibody specific for cyclin D2. Blots were stripped and re-probed sequentially with antibodies specific for cyclin A, cdk6, cdk4 and cdk2. Representative results from 1 among 4 patients studied are shown. **(C)** From the same samples immunoprecipitations were performed with anti-cdk2-specific antiserum agarose conjugate and in vitro kinase reactions were done using Histone H1 as exogenous substrate. Reactions were analyzed by 10% SDS-PAGE, transferred to PVDF membrane and exposed to x-ray film. **(D)** Lysates from the same samples were analyzed by 6% SDS-PAGE, transferred on nitrocellulose membrane and immunoblotted with mAb specific for Rb. **(E-F)** Samples from 2 individual patients were cultured for various time intervals in the presence of IL-7 and examined for the expression of $p16^{INK4}$, $p21^{cip1}$ and $p27^{kip1}$ by immunoblot. Representative results from 2 among 6 patients studied are shown.

IL-7 activates cdk2 and promotes cell cycle progression in T-ALL cells due to downregulation of cyclin dependent kinase inhibitor $p27^{kip1}$.

The enzymatic activation of cdks is regulated by cdk inhibitors, which include members of the kip/cip and the INK4 family. Because IL-7 induced a dramatic increase in the enzymatic activity of the cdks, despite the minimal effect in their protein expression, we examined whether IL-7 influenced the expression of cdk inhibitors. Among these cdk inhibitors, $p21^{cip1}$, $p16^{INK4a}$ and $p27^{kip1}$ were detected. $p27^{kip1}$ was detected in all patients studied (6 out of 6). In contrast, $p16^{INK4a}$ was detected only in 1 patient and $p21^{cip1}$ was detected in 3 patients. When $p16^{INK4a}$ and $p21^{cip1}$ were detectable, they were upregulated during culture with IL-7 (Figure 3E and F). In contrast, $p27^{kip1}$ was significantly downregulated during IL-7 culture in all cases (Figure 3E and F).

To determine whether downregulation of $p27^{kip1}$ had a causative role in IL-7-mediated cell cycle progression, two approaches were undertaken: First, T-ALL cells were cultured with IL-7 in the presence of Rapamycin, which prevents downregulation of endogenous $p27^{kip1}$ [23,24]. Second, T-ALL cells were cultured with IL-7 in the presence of VP22/$p27^{kip1}$ fusion protein, which is capable of translocating recombinant $p27^{kip1}$ into the nucleus of co-cultured cells, thereby leading to forced sustained expression of $p27^{kip1}$ [16]. As shown in Figure 4A, Rapamycin, VP22/$p27^{kip1}$ fusion protein or their combination significantly inhibited IL-7 mediated cell cycle progression and cellular proliferation. Moreover, VP22/$p27^{kip1}$ fusion protein, but not VP22 control protein, inhibited IL-7-induced cdk2 activation (Figure 4B). Consistent with the effects of Rapamycin and VP22/$p27^{kip1}$ on cell cycle progression and cellular proliferation (Figure 4A) and the effect of VP22/$p27^{kip1}$ on cdk2 activation (Figure 4B), analysis of the phosphorylation status of endogenous Rb revealed that Rapamycin, VP22/$p27^{kip1}$ or their combination reversed hyperphosphorylation of Rb induced by IL-7 (Figure 4C). Thus, sustained increased expression of $p27^{kip1}$ prevents IL-7-mediated cdk2 activation, DNA synthesis and progression through the cell cycle. These results strongly indicate that downregulation of $p27^{kip1}$ has a causative role in IL-7 mediated activation of cdk2, resulting in subsequent hyperphosphorylation of Rb and cell cycle progression.

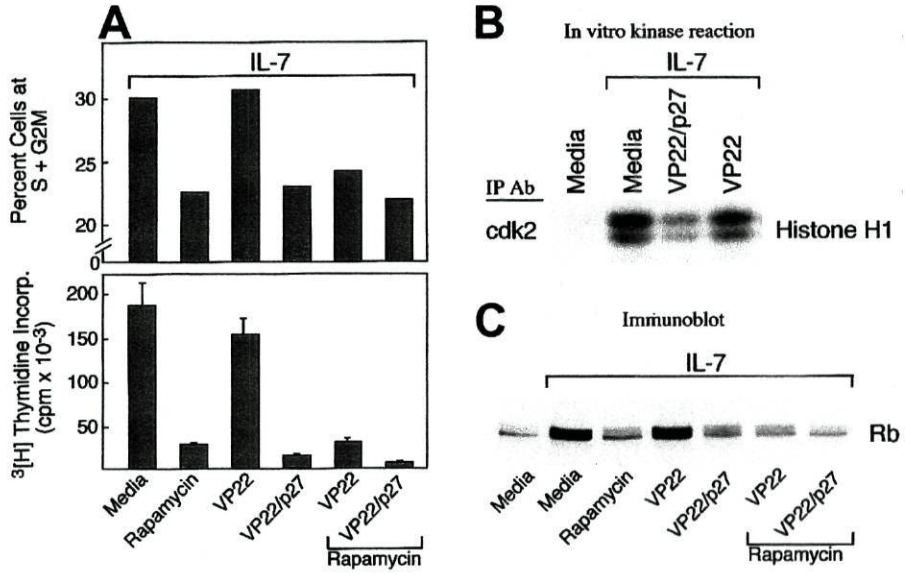

Figure 4. Effects of rapamycin and p27^{kip1}. (A) Rapamycin and forced expression of p27^{kip1} by VP22/p27^{kip1} fusion protein prevent IL-7-mediated increase of T-ALL cells in the S+G2M phase and DNA synthesis. T-ALL cells were cultured with IL-7 either alone or in the presence of Rapamycin, VP22/p27kip1 fusion protein, VP22 control protein or the indicated combinations of the above. Cultures were examined for percentage of cells at the S+G2M phases of the cell cycle and for DNA synthesis by thymidine incorporation as described in Materials and Methods. Representative results from one among three patients studied are shown. **(B)** VP22/p27^{kip1} prevents IL-7-mediated activation of cdk2. T-ALL cells were cultured for 72 hrs under the indicated conditions and activation of cdk2 was determined in cell lysates by in vitro kinase reactions using Histone H1 as exogenous substrate. Reactions were analyzed by SDS-PAGE, transferred to PVDF membrane and exposed to x-ray film. **(C)** Rapamycin, VP22/p27^{kip1} and their combination prevent IL-7-mediated phosphorylation of Rb. T-ALL cells were cultured for 72 hours under the indicated conditions and analyzed by 6% SDS-PAGE, transferred on nitrocellulose membrane and immunoblotted with mAb specific for Rb.

Downregulation of p27^{kip1} is mandatory for induction of Bcl-2 expression and promotion of viability by IL-7 in T-ALL cells.

Propidium iodide staining for analysis of cell cycle progression revealed that addition of Rapamycin, VP22/p27^{kip1} or their combination, not only diminished the ability of IL-7 to induce cell cycle progression but also diminished the ability of IL-7 to promote survival of T-ALL cells during culture (Figure 5A).

Because IL-7 promoted viability of T-ALL cells by upregulating expression of Bcl-2, we examined whether forced sustained expression of p27^{kip1}, which diminished

the ability of IL-7 to promote survival of T-ALL cells, affected expression of Bcl-2. As shown in Figure 5B, VP22/$p27^{kip1}$ alone or in combination with Rapamycin inhibited IL-7-induced upregulation of Bcl-2 protein expression as determined by flow cytometry. These results show that downregulation of $p27^{kip1}$ is mandatory not only for cell cycle progression but also for upregulation of Bcl-2 expression and promotion of viability of T-ALL cells in response to IL-7.

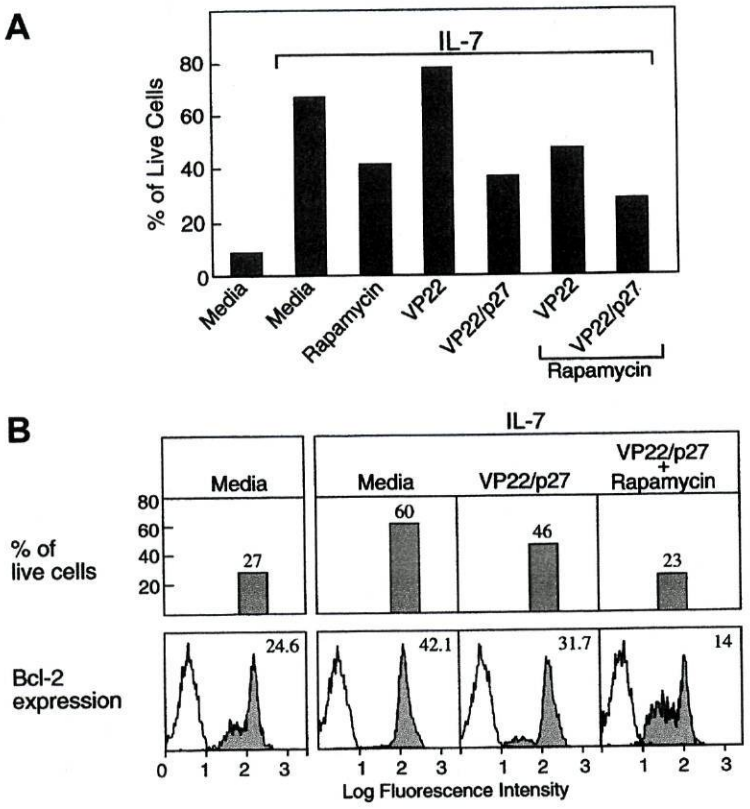

Figure 5. Downregulation of $p27^{kip1}$ is mandatory for induction of Bcl-2 expression and promotion of viability by IL-7 in T-ALL cells. (A) T-ALL cells were cultured for 72 hours under the indicated conditions and the percentage of viable cells was examined by propidium iodide staining followed by FACS analysis as described in Materials and Methods. **(B)** T-ALL cells isolated from the indicated culture conditions were examined for viability as above and for expression of Bcl-2 protein levels by intracellular staining and by FACS analysis, as described in Materials and Methods.

Discussion

IL-7 was initially identified and cloned based on its ability to induce proliferation of B-cell precursors in the absence of stromal cells [25]. However, IL-7 is also expressed in the thymus and has been shown to stimulate the growth of immature double negative and mature single positive thymocytes in thymic organ cultures [6,25-27]. In addition, IL-7/IL-7R signaling plays a critical role in the maintenance of the peripheral T-cell pool, since the few peripheral T-cells seen in the IL-7R-/- mice show impaired response to both TCR-dependent and TCR-independent stimuli due to decreased frequency and clonogenicity of T-cells [2,18]. In humans, defective IL-7R expression results in $T^-B^+NK^+$ severe combined immunodeficiency [28,29]. These data implicate that IL-7 plays a fundamental role at most stages of T-cell development.

Expression of IL-7R during T-cell ontogeny coincides with the expression of Bcl-2 [30] and lack of IL-7 signaling results in reduction of endogenous Bcl-2 levels both in thymocytes and in mature T-cells [18]. Strikingly, the impaired functions of IL7R-/- T-cells are normalized by the introduction of bcl-2 transgene [3,4]. However, although bcl-2 transgene rescued T-cells from the defects of IL-7 deficiency and allowed proliferative response to T-cell mitogenic signals, Bcl-2 itself does not stimulate proliferation and cannot substitute for signals generated during thymic selection driven by TCR/MHC interaction [4]. Furthermore, expression of Bcl-2 inhibits proliferation of stimulated T-cells [31-34]. Therefore, although the IL-7/IL-7R system plays a critical role in the maintenance of Bcl-2 levels in developing thymocytes and peripheral T-cells, the mechanism by which IL-7 induces T-cell proliferation is not mediated by Bcl-2. Our present study shows that IL-7 directly mediates cell cycle progression and proliferation on T-ALL cells by downregulating the cyclin dependent kinase inhibitor $p27^{kip1}$. $p27^{kip1}$ regulates the G1-S transition by stoichiometric inhibition of cyclin E-cdk2 holoenzyme. $p27^{kip1}$ also binds to cyclin D/cdk4 and cyclin D/cdk6 complexes and inhibits their activity, whereas in actively proliferating cells, $p27^{kip1}$ is sequestered by cyclin D-cdk4 complexes and this interaction helps insure that D-type cyclins and E-type cyclins are sequentially activated in the G1 phase [35].

Our present study shows that IL-7 mediates not only upregulation of Bcl-2 and increased survival but also cell cycle progression and proliferation of T-ALL cells. The key step required for the induction of both increased viability and cell cycle progression by IL-7 is the downregulation of $p27^{kip1}$ cdk inhibitor. Downregulation of $p27^{kip1}$ temporally coincided with a dramatic increase in the enzymatic activity of cdk4 and cdk2, hyperphosphorylation of Rb and progression to the S phase of the cell cycle. Downregulation of $p27^{kip1}$ was mandatory for IL-7 mediated cell cycle progression, because Rapamycin, which prevents $p27^{kip1}$ downregulation and forced expression of $p27^{kip1}$ in T-ALL cells inhibited IL-7-mediated cdk2 activation, Rb hyperphosphorylation and cell cycle progression. Moreover, Rapamycin and forced expression of $p27^{kip1}$ also significantly diminished the effect of IL-7 on the promotion of viability by preventing IL-7-mediated upregulation of Bcl-2, suggesting that IL-7-mediated survival is achieved, at least in part, through downregulation of $p27^{kip1}$.

Extensive studies during the last few years provided compelling evidence that $p27^{kip1}$ has a critical role in carcinogenesis, since $p27^{kip1}$ deficient and hemizygote mice develop spontaneous tumors [36]. Moreover, in human tumors the levels of $p27^{kip1}$ protein strongly correlate with prognosis and are controlled by post-transcriptional mechanisms that regulate protein expression in the absence of $p27^{kip1}$ gene mutation [37,38]. Our present studies show that IL-7 induced proliferation of T-ALL cells by downregulating $p27^{kip1}$ protein levels and support the notion that $p27^{kip1}$ might function as a tumor suppressor gene not only because it works as a negative regulator of cell cycle progression but also because it is associated with induction of apoptosis. Studies on IL-7 transgenic mice have shown that IL-7 overexpression promotes malignant transformation of lymphocytes resulting in the development of lymphomas [1]. Based on these findings it has been suggested that, as a biologic effector, IL-7 not only perturbs the development of lymphoid cells but also promotes the growth of such population at risk for malignant transformation [1]. Importantly, IL-7 is produced by bone marrow and thymic stroma [25]. Therefore it is present in the microenvironments in which the malignant T-cells develop and may play an important role in the acquisition of selective growth advantage of the leukemic cells.

Although the molecular pathways of cytokine signaling and their influence on viability and malignant transformation are poorly understood, the early signaling events triggered by engagement of the IL-7 receptors are directly linked to activation of protein tyrosine kinase activity, protein tyrosine phosphorylation, activation of PI3K and inositol phospholipid turnover in human fetal thymocytes and T-lineage ALL blasts [39,40]. PI3K is involved in the regulation of Forkhead family of transcription factors [41], whereas IL-7R ligation directly activates Jak kinases and transcription factors of the STAT family most notably STAT5 [40]. Although most studies have shown that $p27^{kip1}$ expression is mainly regulated at post-transcriptional level by controlling degradation of this protein [42-44], a small number of studies have proposed that $p27^{kip1}$ may also be regulated at the transcriptional level [45,46]. Interestingly, the later study has also implicated the increased expression of $p27^{kip1}$ in the induction of cell death [46]. This mechanism involves PI3K-mediated activation of Forkhead transcription factors, which become phosphorylated and function as repressors of $p27^{kip1}$ transcription. On the other hand, STAT proteins regulate $p27^{kip1}$ expression at a post-transcriptional level and decrease of $p27^{kip1}$ abundance correlates with the ability of cytokines to induce both progression from G1 to the S phase of the cell cycle and malignant transformation [47].

There is extensive evidence that during the G1-S transition $p27^{kip1}$ protein levels change dramatically due to ubiquitin-targeted degradation of $p27^{kip1}$ by the 26S proteasome [43]. Like other G1-S regulatory proteins, $p27^{kip1}$ must undergo phosphorylation on Thr^{187}, which lies within the cdk/MAPK consensus site, in order to be targeted by the ubiquitin ligase complex [48]. Cyclin/cdk holoenzymes can phosphorylate this residue and it has been proposed that cyclin E/cdk2 performs this function *in vivo* [49]. Other studies support a critical role of Ras and the MAPK in the phosphorylation of $p27^{kip1}$ [50]. IL-7 is known to mediate active signaling events by Jak family kinases that are linked to the IL-7 receptor as well as via PI3K, subsequently leading to Ras/MAPK activation [51]. Therefore, IL-7 may regulate direct phosphorylation and ubiquitination of $p27^{kip1}$ leading to its association with other regulatory components of the ubiquitination complex, a process required for subsequent degradation of $p27^{kip1}$.

Further studies will be required in order to elucidate the role of each one of these signaling pathways in IL-7-mediated survival and proliferation of T-ALL cells. Such

studies will determine whether these pathways are equally involved in IL-7 induced downregulation of $p27^{kip1}$ and upregulation of Bcl-2 or whether each pathway has a selective or predominant role in regulating these two downstream molecular events. Regardless of the mechanism, our results show for the first time that a causative link between IL-7-mediated proliferation and $p27^{kip1}$ downregulation exists in malignant T-cells. More importantly, sustained increase of $p27^{kip1}$ expression in T-ALL cells not only prevents their proliferation but also promotes apoptosis by preventing IL-7-mediated upregulation of Bcl-2. Further understanding of the mechanisms involved in this process could lead to improved therapeutic strategies aimed to achieving high levels of $p27^{kip1}$ expression in T-ALL blasts perhaps with a gene therapy approach or by the use of cdk inhibitors that are currently being broadly developed [52].

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Chapter 4

AN IL-7-DEPENDENT HUMAN LEUKEMIA T-CELL LINE AS A VALUABLE TOOL FOR DRUG DISCOVERY IN T-ALL

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Submitted

Abstract

The specific targeting of critical signaling molecules may provide efficient therapies for T-cell acute lymphoblastic leukemia (T-ALL). However target identification and drug development are limited by insufficient numbers of primary T-ALL cells and by their high rate of spontaneous apoptosis. Therefore, we established an IL-7-dependent T-ALL cell line – TAIL7 – that maintains several biological and signaling properties of its parental leukemia cells. TAIL7 cells are pre-T ALL cells that proliferate in response to IL-7 and IL-4. IL-7-stimulation of TAIL7 cells prevents spontaneous apoptosis, and induces cell activation and cell cycle progression. The signaling events triggered by IL-7 include downregulation of the cdk inhibitor $p27^{kip1}$ and hyperphosphorylation of Rb. Activation of TAIL7 cells by IL-7 also leads to phosphorylation of STAT5 and Erk1/2. Importantly, specific blockade of Jak3 by its inhibitor WHI-P131 abrogates IL-7-mediated proliferation and survival of TAIL7 cells, suggesting that activation of Jak3 may be a critical event in T-ALL biology. Since TAIL7 cells seem to be a biologically accurate surrogate for primary leukemia T cells, they may constitute a valuable tool for the study of the signaling pathways implicated in T-ALL. Exploitation of this cell line may allow the identification of molecular targets, and allow for the rational design and validation of anti-leukemia signaling-inhibitors.

Introduction

Despite the considerable success of current therapies for childhood T-cell acute lymphoblastic leukemia (T-ALL), disease relapse, treatment-related complications represent considerable challenges. More effective and less toxic therapies are necessary to improve outcome and the therapeutic index. A legitimate approach may be to target molecules and pathways that play critical roles in the biology of the tumor, a strategy validated by the success of STI-571 in chronic myelogenous leukemia and by the promising results of clinical trials with other signaling inhibitors [1-3]. An inherent obstacle is the availability of malignant T-cells in numbers sufficient to perform both the signaling experiments necessary for target identification, drug screening and pre-clinical validation.

We have previously shown that interleukin-7 (IL-7) promotes the survival and proliferation of T-ALL cells by modulating Bcl-2 expression and cell cycle progression through downregulation of the cdk inhibitor $p27^{Kip1}$ [4]. IL-7 has been implicated in normal T-cell survival and proliferation, and, in immature T-cells, plays a non-redundant anti-apoptotic role by upregulating Bcl-2 expression [5]. Mice deficient in IL-7 or lacking IL-7 receptor, are lymphopenic due to a defect in T-cell expansion at an early stage of differentiation, and the few mature T-cells that develop are functionally impaired [5-7]. Importantly, IL-7 has been implicated in leukemogenesis since IL-7 transgenic mice develop lymphoid malignancies [8]. Malignant T-ALL cells express functional IL-7 receptors [9], and IL-7 stimulates blast colony formation and DNA synthesis, suggesting that it may play an important regulatory role in the pathophysiology of T-ALL [9,10]. Finally, IL-7 is produced in the bone marrow (BM) and thymic stroma [11] and, therefore, is present in the microenvironments where malignant T-cells develop.

In an effort to develop better tools for anti-leukemia target validation in T-ALL, we have established a **T**-cell **A**cute-Leukemia **IL-7**-dependent cell line (TAIL7) derived from a 7-year old male patient. After more than 8 months in continuous culture, TAIL7 cells maintain several features from the patient's primary malignant cells, including: 1) the monoclonal TCR-γ rearrangement; 2) the immunophenotype of T-cell blasts arrested at an immature thymocyte stage of maturation – pre-T ALL; 3) the proliferative response to IL-7 and IL-4; 4) the engraftment and establishment of leukemia in NOD/SCID mice,

and, importantly; 5) the signaling events triggered by IL-7 stimulation, namely downregulation of $p27^{kip1}$, and hyperphosphorylation of the retinoblastoma protein (Rb). Finally, using a JAK3-specific inhibitor, we demonstrate that blockade of signaling pathways triggered by IL-7 significantly abrogates proliferation and survival of the TAIL7 cells. Our results indicate that TAIL7 may constitute a biologically-relevant tool for the identification of molecular targets and the development of novel therapeutic agents for T-ALL.

Materials and Methods

T-cell ALL Specimen. Primary cells were obtained from the peripheral blood of a 7-year old male patient diagnosed with T-ALL. Informed consent and Institutional Review Board approval were obtained using federal guidelines. The specimen contained >95% of leukemic T-cells with an immature phenotype: $CD1^-$ $CD2^+$ $CD5^+$ $CD7^+$ $CD3^-$ $CD4^+$ $CD8^+$.

Establishment and Maintenance of TAIL7 cell line. Primary cells were thawed, separated by density centrifugation, and cultured in 24-well plates at $2x10^6$ cells/ml in RPMI-1640 supplemented with 10% FBS (RPMI-10) and 10ng/ml of recombinant IL-7 (Endogen, Woburn, MA). After 9 days in culture at 37°C, 5% CO_2, dead cells were removed using the MACS Dead-cell Removal® kit (Miltenyi Biotec, Auburn, CA), and viable cells recultured under similar conditions. This strategy was employed for the initial three cycles of leukemic T-cell culture and IL-7-mediated expansion. For further passages, every 7 to 9 days, viable leukemic cells were separated by density centrifugation, and replated in IL-7. To confirm IL-7-responsiveness and dependency, viability and proliferation assays were performed periodically. TAIL7 cells have been maintained on IL-7 continuous culture for more than 8 months. When necessary, viable TAIL7 cells were IL-7-deprived in either RPMI-10 for 5-10 days or RPMI without FBS for 2-3 days prior use. For signaling experiments, cultured cells were washed twice with PBS and incubated for 15 min. at 37°C with pre-warmed PBS alone or with indicated

cytokines. The reaction was stopped by placing samples on ice and adding ice-cold PBS. Cells were washed twice with cold PBS and whole cell lysates prepared.

Immunophenotype. Primary leukemic cells and TAIL7 cells were phenotyped using standard methodology [12]. The fluorochrome-conjugated mAbs against the surface antigens shown in Table 1 were obtained from BD Pharmingen (San Diego, CA) and Beckman-Coulter (Miami, FL). Samples were analyzed in a Coulter Elite or XL flow cytometer (Beckman-Coulter), and data were acquired as listmode files. At least 5,000 positive events were acquired for each sample.

Clonality Assessment. The characterization of immunoglobulin heavy chain (IgH) and T-cell receptor (TCR) gene rearrangements on the primary leukemic and TAIL7 cells was performed using previously described primers and protocols [13,14]. Sequencing was performed using the BigDye Terminators Cycle Sequencing kit (Applied Biosystems, Foster City, CA). V, D, and J segments were identified using the ImMunoGeneTics (IMGT) database [15].

Engraftment into NOD/SCID Mice. To assess tumorigenicity, viable TAIL7 cells ($5x10^6$ cells) were transplanted IV into irradiated NOD/SCID mice. Animals were sacrificed when moribund. Blood was collected via orbital puncture and bone marrow was extracted by flushing of long bones. Femurs, spleen, liver, kidneys and, when possible, thymus and lymph nodes were collected for histological analysis.

Proliferation assays. TAIL7 cells were cultured in flat-bottom 96-well plates at $4x10^5$ cells/well, in standard RPMI-10 or in the presence of IL-7 (10ng/ml), IL-2 (100U/ml), IL-4 (10ng/ml), IL-9 (50U/ml), IL-15 (20ng/ml) or PHA (1μg/ml) + PMA (1ng/ml). Cultures were performed in triplicates for the indicated time points. To assess DNA synthesis, ^{3}H-Thymidine (1μCi/well) was added for 16 hours prior cell harvest, and measured using a liquid scintillation counter. Proliferation index (P.Index) was calculated as: P.Index = (mean cpm for each experimental condition) / (mean cpm for medium alone).

Cell Viability and Activation. Quantitative determination of cell viability was performed using an Annexin V-based apoptosis assay (R&D Systems, Flanders, NJ). Briefly, cells were suspended in the appropriate binding buffer, stained with FITC-conjugated Annexin V and propidium iodide (PI) at room temperature for 15 minutes, and subsequently analyzed by flow cytometry. Since activated cells have an increased size and light refraction, activation status was assessed by measuring changes in these physical parameters in viable TAIL7 cells (Annexin V- and PI-negative).

Cell Cycle Analysis. Cell cycle distribution was performed by assessment of DNA content using a PI staining. Cells were resuspended in PBS and fixed with ice-cold 80% ethanol for at least 30 minutes. PI (2.5mg/ml) and ribonuclease A (50mg/ml) were added and samples incubated in the dark, for 30 min at 37°C. Flow cytometric analysis was performed using Lysis II (Becton Dickinson, Mountain View, CA) or Coulter's XL2 software, and analysis of cell cycle histograms was carried out using ModFit LT (Verity, Topsham, ME) or WinCycle DNA Analysis software (Phoenix Flow Systems, San Diego, CA).

Immunoblotting. Equal amounts of protein (50μg/sample) were analyzed by 10% SDS-PAGE or 6% SDS-PAGE (for Rb phosphorylation), transferred onto nitrocellulose membranes, and immunoblotted with the indicated monoclonal antibodies: $p27^{kip1}$ (BD Transduction Laboratories, Lexington, KY); Phospho-Erk mAb (Santa Cruz Biotechnology, Santa Cruz, CA); Rb (BD Pharmingen); ZAP-70, phospho-tyrosine (clone 4G10) and phospho-STAT5 (Upstate Biotechnology, Lake Placid, NY). Immunodetection was performed by incubation with HRP-conjugated anti-mouse IgG (1:5,000) (Promega, Madison, WI) and developed by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Stripping and reprobing of the immunoblots were done as described [16].

Evaluation of JAK3-Specific Inhibitor. Effect of the JAK3 Inhibitor WHI-P131 (Calbiochem, San Diego, CA) on TAIL7 cells was evaluated in proliferation and viability

studies, as described above. WHI-P131 was used at final concentrations of 50, 100 and 200 μM.

Real-time Quantitative PCR. Real time quantitative RT-PCR analysis was performed as previously described [17]. Briefly, *HOX11, HOX11L2, TAL1, TAL2, LMO1, LMO2, BHLHB1* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels were analyzed in parallel experiments using 100 ng of RNA extracted from the cell lines analyzed. Real-time RT PCR reactions were performed in an ABI PRISM 7700 Sequence Detection System instrument (Applied Biosystems). RNA copy numbers were calculated using standard curves consisting of serial 10-fold dilutions from 10^7 to 10^2 copies of in-vitro transcribed RNA. Samples containing less than 10^6 copies of *GAPDH* message per 100 ng of RNA were considered to have poor quality and were discarded from further analysis. PCR analysis of the *Tal1d* variant resulting from a 90Kb deletion in chromosome band 1p32 adjacent to the *TAL1* locus was performed in genomic DNA extracted from the cell lines analyzed. [18]

TAIL7 Fingerprint. For DNA profiling of TAIL7 cells, characterization of several loci of variable number of tandem repeats (VNTR) and short-tandem repeats (STR) was performed. For VNTR profiling, TAIL7 genomic DNA was digested with Hae III, electrophoresed in a 1% agarose gel, transferred to Biodyne A membrane (Pall Life Sciences, Ann Arbor, MI), and sequentially hybridized with one of seven different alkaline phosphatase-conjugated oligonucleotide probes: MS1 (D1S7), PH30 (D4S139), LH1 (D5S110), CEB42 (D8S358), D17S79 (Invitrogen Life Technologies, Carlsbad, CA), D2S44, or D10S28 (LifeCodes, Stamford, CT); plus the ACES 2.0 Marker Probe Plus (Invitrogen) according to manufacturer's instructions. DNA from the well-characterized K562 cell line was included as control. The size of the "alleles" was calculated using the Kodak 1D software (Eastman Kodak Company, Rochester, NY). For STR profiling, 18 loci fragments were amplified in multiplexed PCR reactions using fluorescently labeled primers, and the length of the individual-specific "alleles" was determined in an ABI 377 DNA Sequencer (Applied Biosystems).

Results

Establishment of an IL-7-Dependent Leukemic T-cell Line

A very high rate of spontaneous apoptosis and lack of significant *in vitro* expansion of leukemic T-cells represent considerable obstacles to both the study of the biology of T-ALL and the evaluation of novel therapeutic agents. To circumvent such limitations, we attempted to establish a leukemic T-cell line that could be expanded by *in vitro* culture while maintaining the biological and signaling properties of primary leukemic cells. We have selected IL-7 as the stimulus for the leukemic cells, based on its *in vitro* effects on T-ALL cells [4,9,10,19], its presence in the leukemia microenvironment and its putative role in leukemogenesis. An IL-7 responsive T-cell line – TAIL7 – was derived from leukemic cells obtained from the peripheral blood of a 7-year old male patient, with >95% of T-cell blasts at diagnosis. Primary cells were cultured in the presence of 10ng/ml of recombinant IL-7 for periods of 7-9 days as described in Material and Methods. TAIL7 cells have been in continuous culture in IL-7 for more than 8 months. The average 7-day expansion rate of TAIL7 cells is 3.1 ± 0.5 fold (n=17 determinations). Viability and proliferation assays were performed routinely and showed that TAIL7 cells maintain their responsiveness to IL-7 and progressively die out in its absence (data not shown).

TAIL7 Cells Are Immature T-ALL Cells Sharing the Phenotype and Clonality of Their Parental Leukemic Cells

To determine whether TAIL7 cells replicate the patient's primary leukemic T-cells, immunophenotypic and clonality analyses were performed in the parental leukemic cells and TAIL7 cells from various culture time points (1, 2, 4 and 6 months of continuous IL-7 culture). Primary leukemic and TAIL7 cells share an identical phenotype as $CD7^+$ $CD2^+$ $CD5^+$ $CD3^-$ $CD1a^-$ cells and lack B-cell, NK-cell and myeloid cell markers (Table 1), which is consistent with an immature thymocyte [20] or pre-T ALL lymphoblast [21] phenotype. TAIL7 cells also express CD4 and CD8 and, consistent with their lack of CD3, are negative for both TCR$\alpha\beta$ and TCR$\gamma\delta$. Further evidence of their staging as pre-T ALL lymphoblasts is their lack of expression of CD34 and CD45RA,

and positivity for CD45RO [22,23]. Finally, analysis of TAIL7 cells at different culture time points showed that no significant phenotypic changes occurred, thus suggesting that continuous exposure to IL-7 did not induce cell differentiation.

Table 1. Immunophenotype of parental primary T-ALL cells and TAIL7 cell line

	Primary cells	TAIL7
CD1a	-	-
CD2	+++	+++
CD3	-	-
CD4	++	++
CD8	++	+++
CD5	+++	+++
CD7	+++	+++
TCRαβ	-	-
TCRγδ	-	-
MHC I	+++	+++
MHC II	-	-
CD25	-	-
CD44	++	++
CD45RA	-	-
CD45RO	+++	+++
CD34	-	-
CD38	+++	+++
CD13	-	-
CD19	-	-
CD33	-	-
CD56	-	-

Expression of each surface antigen was classified according to the percentage of positive cells as compared to the isotype control. - <20%; + 20 to 40%; ++ 40 to 70%; +++ >70%.

The presence of TCR and IgH rearrangements was investigated to ascertain the clonality of TAIL7 cells and their parental T-ALL cells. An extensive series of putative TCRδ, TCRγ, TAL1, and IgH junctional rearrangement regions were PCR-amplified and subjected to heteroduplex analysis. A single monoallelic TCRγ rearrangement (VγI-Jγ1.3/3.2) was observed on both the TAIL 7 cells and the primary leukemic cells (Table 2) thus excluding the occurrence of subclone derivation or ongoing recombination on TAIL7 cells.

Table 2. TAIL7 cells and their parental T-ALL cells are clonally identical.

Day	TCR γV8*01	N region	TCR γJ2*01
0	5'...TATTACTGTGCCACCTGGGA----	CACCTC	GAATTATTATAAGAAACTCTTTGGCA...3
26	5'...TATTACTGTGCCACCTGGGA----	CACCTC	GAATTATTATAAGAAACTCTTTGGCA...3
>70	5'...TATTACTGTGCCACCTGGGA----	CACCTC	GAATTATTATAAGAAACTCTTTGGCA...3

The presence of TCR and IgH rearrangements was investigated to evaluate the clonality of TAIL7 cells and their parental T-ALL cells. An extensive series of putative TCRδ, TCRγ, TAL1, and IgH junctional rearrangement regions were PCR-amplified and subjected to heteroduplex analysis. A single monoallelic TCRγ rearrangement (VγI-Jγ1.3/3.2) was observed on both the TAIL 7 cells and the primary leukemia cells.

IL-7 Induces TAIL7 Cell Proliferation, Activation, Survival and Cell Cycle Progression

We next evaluated whether TAIL7 cells maintain the pattern of responsiveness to IL-7 and other γ_c-signaling cytokines observed for their parental primary leukemic cells. TAIL7 cells showed a robust proliferative response to IL-7 and a weaker response to IL-4 (Figure 1A). No proliferation was observed in response to IL-2, IL-9 or IL-15. No synergisms on TAIL7 cell proliferation were induced by any combination of these cytokines (data not shown). Also, TAIL7 cells fail to proliferate when cultured with PHA plus PMA, instead showing an inhibitory effect (Figure 1A). Importantly, the reactivity of TAIL7 cells to these cytokines and mitogens was in absolute concordance with the pattern observed for the parental leukemic cells from which this cell line was derived (Figure 1B). Expectedly, IL-7-promoted proliferation of TAIL7 correlated with an increased percentage of cells in S- and G2/M phases of the cell cycle (Figure 2A), and resulted in an increased cell size and complexity, as illustrated by the physical changes detected by flow cytometry (Figure 2B; FSC *versus* SSC signals). Finally, stimulation of TAIL7 cells with IL-7 promoted cell viability, which was more pronounced under more stringent pre-culture starvation conditions (Figure 2C). Again, these findings are consistent with observations using the patient's tumor cells, although the survival effect of IL-7 on the parental leukemic cells was more pronounced that on the cell line (average

30% increase on viability at 96 hours *versus* 17% for the TAIL7 cells). To confirm the specific ability of IL-7 to promote long-term T-ALL cell survival and expansion, we tried to establish a cell line from the primary cells of the same patient using 10ng/ml IL-4 (Endogen). Although IL-4 induces short-term proliferation of both TAIL7 and parental leukemic cells, we were not able to establish an IL-4-dependent cell line, since the cells progressively died even in the presence of the cytokine.

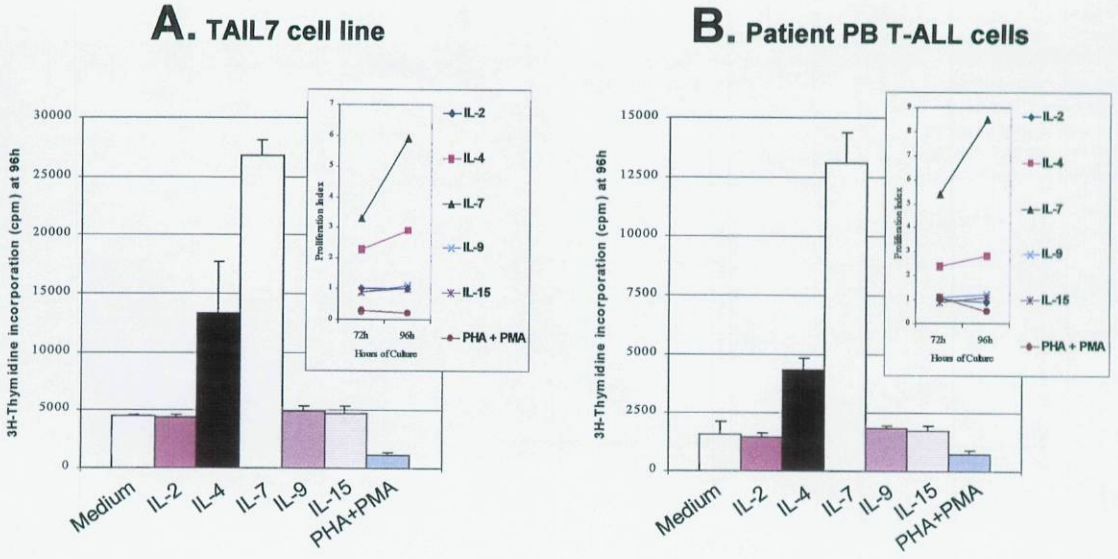

Figure 1. TAIL7 cells and their parental leukemia T-cells proliferate in response to IL-7 and IL-4. TAIL7 cells **(A)** and the patient's peripheral blood blasts **(B)** were cultured for 96 hours in medium alone or the indicated cytokine or mitogen, and proliferation was measured by ^{3}H-thymidine incorporation. Insets: Proliferation index (see Materials and Methods) for each condition at 72 and 96 hours. Results are representative of 2 independent experiments for the primary leukemia cells and 3 independent experiments for the TAIL7 cell line.

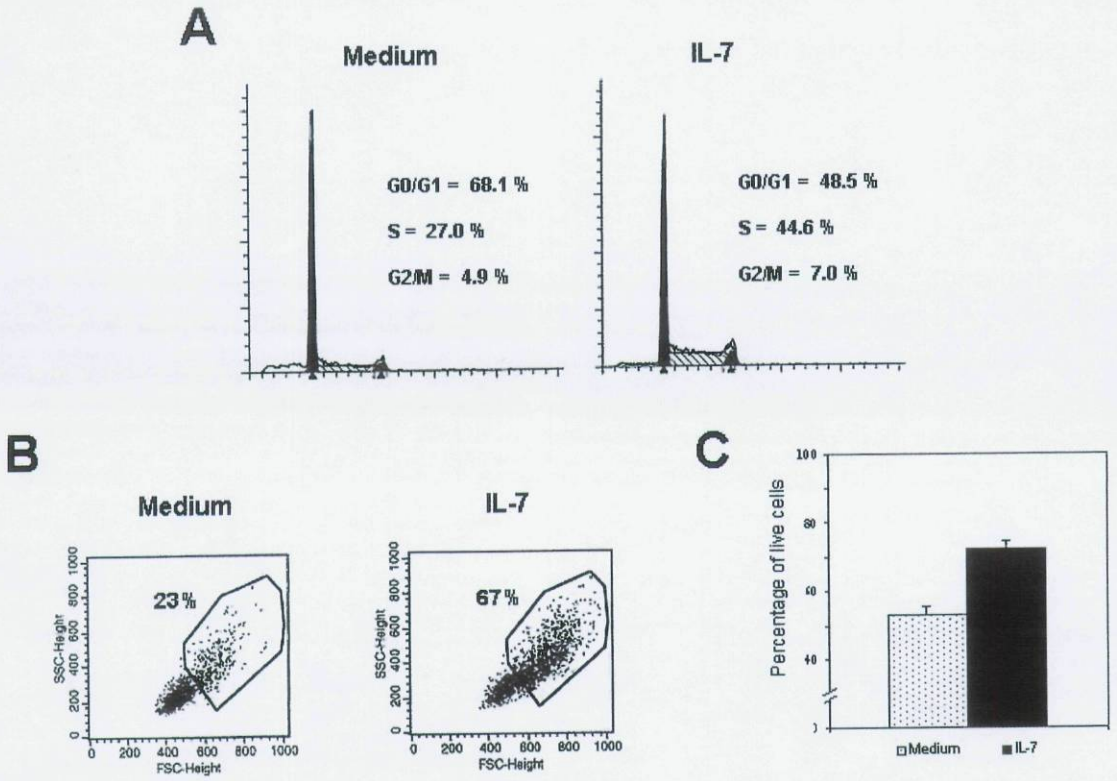

Figure 2.IL-7 activates TAIL7 cells, and promotes cell cycle progression and increased viability. (A) TAIL7 cells were cultured for 72 hours with or without IL-7 and their cell cycle distribution was examined by PI staining. Results are representative of three independent experiments. **(B)** At 72 hours, activation was assessed by FACS analysis, by gating the live cell population and measuring the changes in cell size (FSC) and complexity (SSC). The 'activation' shown was defined considering the distribution of the control population (medium alone). Similar results were obtained in seven independent experiments. **(C)** At 96 hours, cells were stained with Annexin V-FITC/ PI and the percentage of viable cells assessed by FACS analysis. Results are expressed as mean and standard deviation of three samples for each condition and are representative of five independent experiments.

TAIL7 Cells Are Tumorigenic

To determine tumorigenicity, TAIL7 cells were transplanted IV into irradiated NOD/SCID mice ($5x10^6$ viable cells/mice). In all animals tested (n=6), TAIL7 cells engrafted and developed into a leukemia involving multiple organs. Animals were moribund and sacrificed at 64.3±2.3 days after cell injection (range 63 - 67 days). Histological analyses showed that, in all animals, the BM was packed with leukemic cells (Figure 3A, B). Splenomegaly and enlarged lymph nodes were a common occurrence in the transplanted animals with large numbers of leukemic cells present in the spleen and lymph nodes (Figure 3C, D, E). Tumor cells were also observed in the liver and kidneys (Figure 3F, G). Importantly, the time-span of TAIL7 growth and leukemia development in NOD/SCID mice was more consistent to that observed for primary leukemic cells than that of established, stimuli-independent leukemic cell lines (data not shown).

IL-7-Triggered Signaling Events on TAIL7 Cells

To further investigate whether the TAIL7 cell line is a reliable substitute for primary leukemic cells, we have compared the signaling events triggered by IL-7 stimulation on both TAIL7 cells and their parental leukemic cells. This is particularly important as TAIL7 cells are to be employed as surrogate 'primary' leukemic T-ALL cells for the identification of signaling molecules that may constitute therapeutic targets.

First, we evaluated the impact of IL-7 on the expression of the cell cycle regulator $p27^{kip1}$, since we have previously demonstrated that IL-7 induces cell cycle progression in T-ALL cells through down-modulation of the activity of this cdk inhibitor [4]. As shown in Figure 4A, we observed a marked downregulation of $p27^{kip1}$ in TAIL7 cells following IL-7 stimulation. We have also observed that IL-4, the other cytokine that induces TAIL7 proliferation, also leads to a similar decrease in $p27^{kip1}$ expression (data not shown). IL-7 also induced upregulation of the hyperphosphorylated form of the Retinoblastoma protein (Rb), a critical substrate of cyclin/cdk activity, indicating that cells are committed to cell cycle progression (Figure 4B). An identical pattern of IL-7-mediated $p27^{kip1}$ downregulation (Figure 4C) and hyperphosphorylation of Rb (data not shown) was found in parental primary leukemic cells.

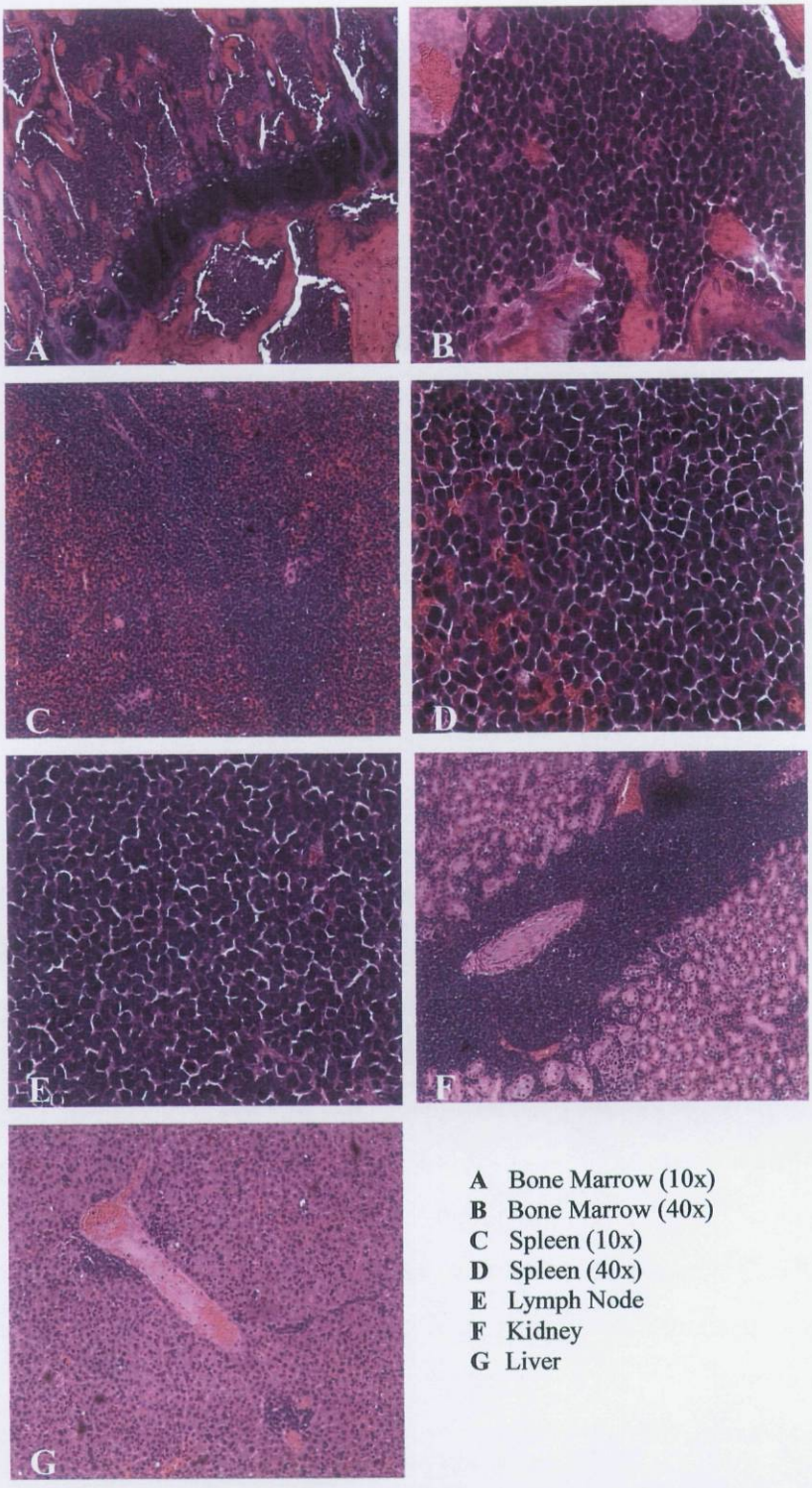

Figure 3. TAIL7 cells engraft NOD/SCID mice and are tumorigenic. Viable TAIL7 cells were transplanted into NOD/SCID mice ($5x10^6$ cells/animal). Mice were sacrificed when moribund and the indicated organs collected for histological analysis.

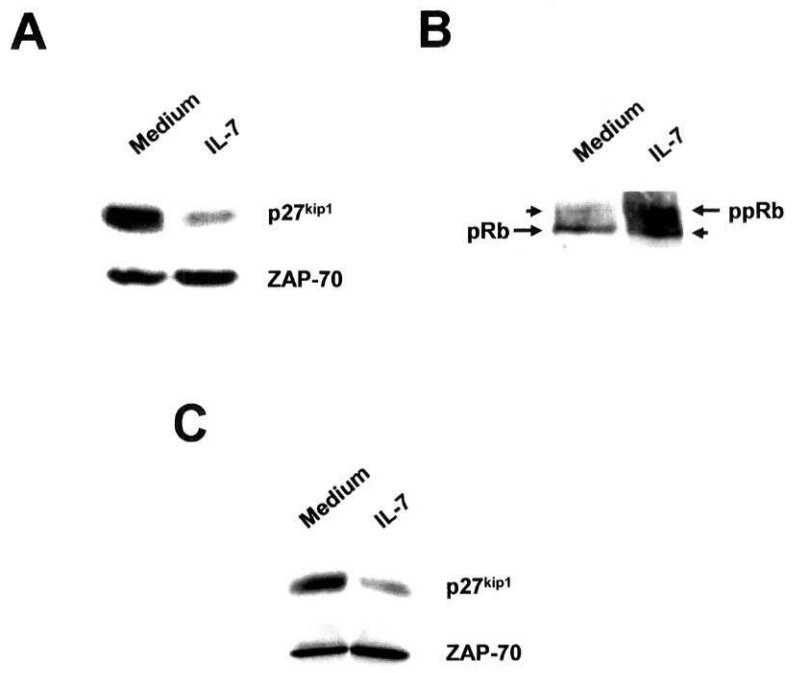

Figure 4. IL-7 downregulates $p27^{kip1}$ expression and induces hyperphosphorylation of Rb in TAIL7 cells. (A) Lysates of TAIL7 cells cultured for 96 hours were resolved by 10% SDS-PAGE and immunoblotted with a $p27^{kip1}$-specific antibody. Membrane was stripped and reprobed for ZAP-70 to demonstrate equal total protein loading. **(B)** Lysates were analyzed by 6% SDS-PAGE and immunoblotted for Rb. The antibody recognizes both hypo- (pRb) and hyper-phosphorylated (ppRb) forms of Rb. Representative results of three independent experiments are shown. **(C)** Lysates of parental primary leukemia cells cultured for 72 hours were resolved by 10% SDS-PAGE and sequentially immunoblotted with p27kip1 and ZAP-70 antibodies.

Second, since protein tyrosine kinases are essential mediators of most intracellular signaling pathways, we evaluated the pattern of tyrosine phophorylation triggered by IL-7 on TAIL-7 cells. Immunoblot with an anti-phosphotyrosine monoclonal antibody (4G10) showed that IL-7 stimulation induced phosphorylation of at least three different bands (Figure 5A). It also revealed that two additional bands (~60-70 kD) were markedly and constitutively tyrosine-phosphorylated (Figure 5A; dashed arrows). Additional studies showed that the two higher molecular weight bands were JAK3 (~120 kD) and STAT5 (~94 kD), which are known targets of IL-7-mediated signaling on normal T-cells (data not shown). To confirm that the JAK/STAT pathway was activated on TAIL7 cells in response to IL-7 we used a STAT5 a/b-phospho-specific antibody that recognizes the

phosphorylated STAT5-residues Y694 and Y699. Results showed a striking upregulation of phospho-STAT5 upon IL-7 stimulation (Figure 5B) that had been somewhat under-estimated using the 4G10 antibody. Similarly, phosphorylation of p44 MAPK (Erk 1) and p42 MAPK (Erk 2) was also triggered by IL-7 stimulation of TAIL7 cells (Figure 5B).

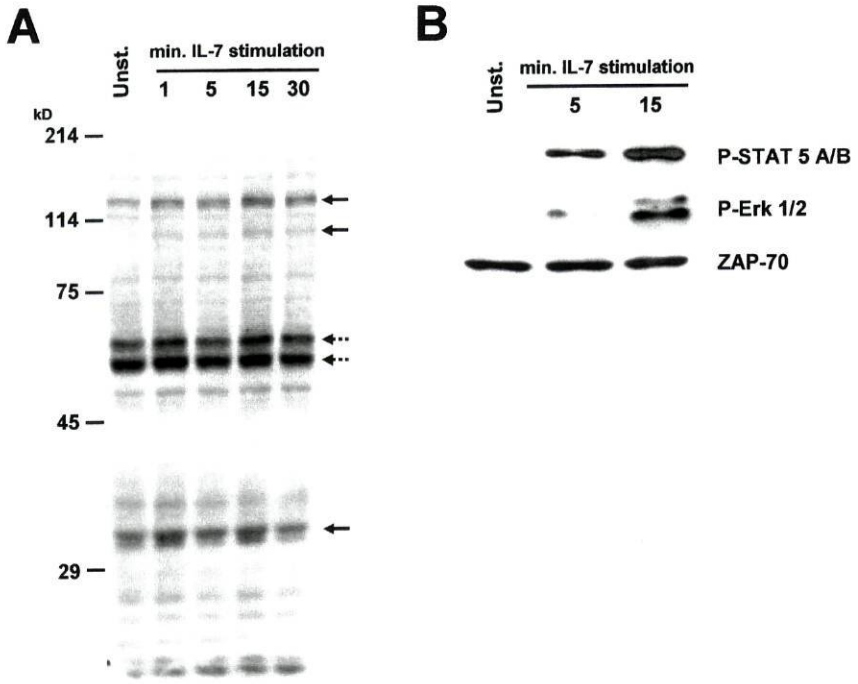

Figure 5. IL-7 stimulation induces tyrosine phosphorylation of different molecules and activates JAK/STAT and ras/MAPK pathways in TAIL7 cells. TAIL7 cells were stimulated with 50 ng/ml IL-7 at 37°C for the indicated periods or left unstimulated (Unst.), and lysates were analyzed by 10% SDS-PAGE. **(A)** Proteins phosphorylated at tyrosine residues were detected by immunoblotting with a phosphotyrosine-specific antibody (4G10). Apparent molecular weight is shown in kilodalton (kD) on the left. Arrows on the right denote the bands that showed increased tyrosine phosphorylation after IL-7 stimulation. Dashed arrows indicate bands that were markedly tyrosine-phosphorylated in unstimulated cells. **(B)** Phosphorylated STAT5 and Erk were probed with phospho-specific antibodies or ZAP-70 loading control. P-STAT5 recognizes Y694 and Y699 of STAT5a and STAT5b. P-Erk recognizes Y204 of Erk 1 and 2. Results are representative of three independent experiments.

Significantly, the cell cycle-related events triggered by IL-7 stimulation of TAIL7 cells are absolutely consistent with those observed using the parental leukemic cells and specimens from other T-ALL patients [4]. Moreover, the signaling pathways and the functional responses activated by IL-7 stimulation of TAIL7 cells and primary leukemic

cells seem to be equivalent (Chapter 5). These findings suggest that the TAIL7 cells may serve as a valid tool to study the signaling pathways that are implicated in the pathophysiology of T-ALL and the rational design of anti-leukemia signaling-inhibitors.

The JAK3-Specific Inhibitor WHI-P131 Abolishes the IL-7-Promoted Proliferation and Survival of TAIL7 Cells

Since the JAK/STAT pathway is activated by IL-7 stimulation of TAIL7 cells (Figure 5B), we evaluated the effect of the JAK3-specific inhibitor WHI-P131 on TAIL7 cell survival and proliferation. WHI-P131 completely abrogates the stimulatory effect of IL-7 on TAIL7 cells, inhibiting both cell proliferation (Figure 6A; with optimal dose of 100 μM) and survival (Figure 6B). The effect of WHI-P131 on both TAIL cell proliferation and survival was dose-dependent (data not shown) and demonstrated that the specific blockade of JAK3 effectively abrogates the stimulatory effect of IL-7.

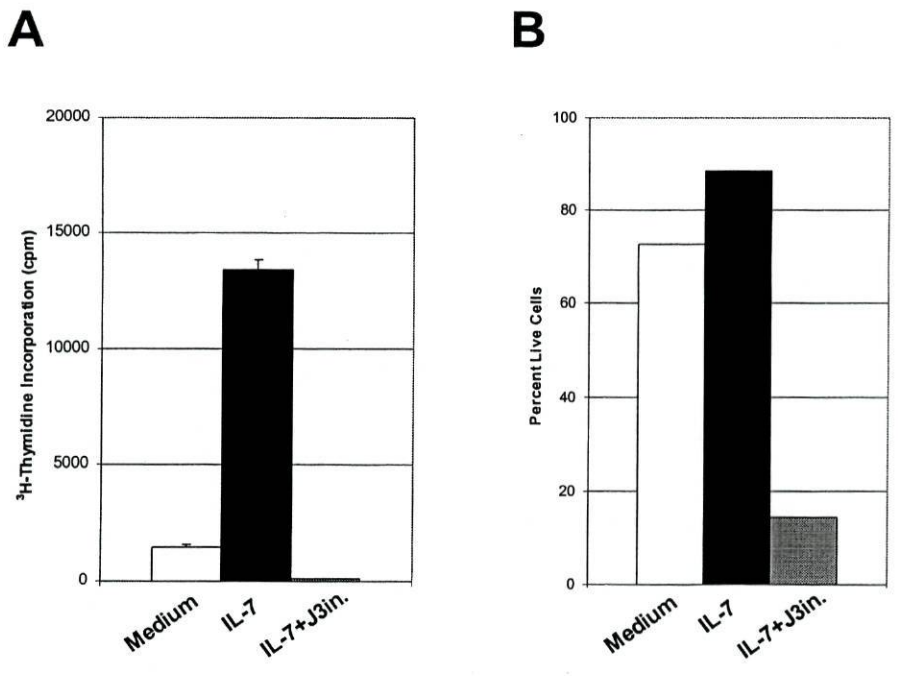

Figure 6. JAK3-Inhibitor WHI-P131 abrogates IL-7-induced proliferation and viability in TAIL7 cells. TAIL7 cells were cultured for 72 hours in medium alone, in the presence of IL-7 or with IL-7 plus 100 μM of WHI-P131 (J3In.). **(A)** Proliferation was examined by ^{3}H-Thymidine incorporation. **(B)** Viability was assessed by Annexin V-FITC / PI staining and FACS analysis.

Importantly, the effect of WHI-P131 on TAIL7 cells mimics its inhibitory activity on the IL-7-promoted proliferation of primary tumor cells from T-ALL patients (Chapter 2). As shown in Figure 5, the WHI-P131-mediated marked inhibition of TAIL7 cells surpassed that of absence of IL-7 thus suggesting that some degree of constitutive activation of JAK3 exists in TAIL7 cells. This finding is in agreement with the basal level of JAK3 tyrosine phosphorylation observed in unstimulated TAIL7 cells, which was upregulated by IL-7 (Figure 5A; top arrow).

T-cell Oncogene Expression and DNA Fingerprinting of TAIL7 Cells

To further characterize TAIL7 cells, we have studied the expression of the T-cell oncogenes *TAL1, LYL1, LMO1, LMO2, HOX11 and HOX11L2*, which have been shown to be aberrantly expressed in T-cell leukemia [17,24]. TAIL7 cells express high levels of *TAL1*, which are similar to those observed in JM-Jurkat cells and significantly higher than in cell lines expressing oncogenic levels of TAL1 resulting from the presence of Tal1d rearrangement (Figure 7). TAIL7 cells also express high levels of *LMO2* and intermediate levels of *LYL1*. We have not observed detectable expression of *LMO1* (Figure 7), *HOX11* or *HOX11L2* (data not shown) on TAIL7 cells.

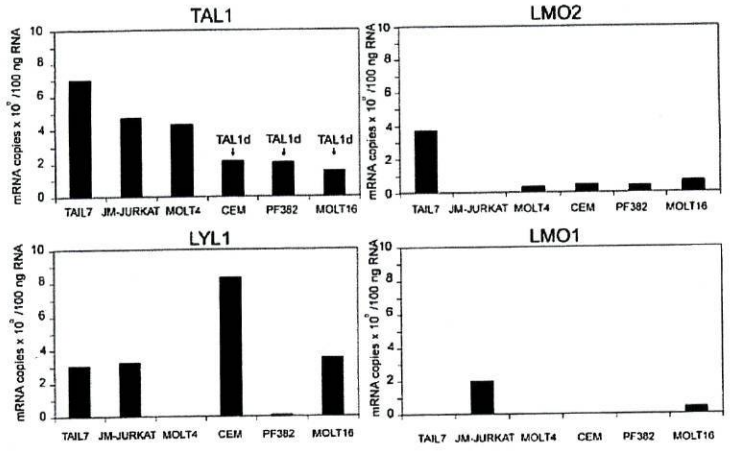

Figure 7. TAIL7 cells express the oncogenes TAL1, LMO2 and LYL1. Expression of the T-cell oncogenes TAL1, LMO1, LMO2, LYL1, HOX11 and HOX11L2 was determined using quantitative RT-PCR analysis of TAIL7 cells and five immortalized T-cell leukemia lines. PCR analysis of the *Tal1d* variant was performed as described.

Finally, to establish a genetic "identity" of the TAIL7 cell line, a DNA profile was performed using validated fingerprint techniques of different single- and multiple locus variable-numbers of tandem repeats (VNTR) and short tandem repeats (STR). The results are presented in Table 3. This DNA fingerprint may be useful for future genetic authentication of this cell line and resolve potential problems of cell line misidentification and contamination, occurrences that have been shown to affect various cell lines thus hampering the usefulness and accuracy of several studies [25].

Table 1. Immunophenotype of parental primary T-ALL cells and TAIL7 cell line

	Primary cells	TAIL7
CD1a	-	-
CD2	+++	+++
CD3	-	-
CD4	++	++
CD8	++	+++
CD5	+++	+++
CD7	+++	+++
TCRαβ	-	-
TCRγδ	-	-
MHC I	+++	+++
MHC II	-	-
CD25	-	-
CD44	++	++
CD45RA	-	-
CD45RO	+++	+++
CD34	-	-
CD38	+++	+++
CD13	-	-
CD19	-	-
CD33	-	-
CD56	-	-

Expression of each surface antigen was classified according to the percentage of positive cells as compared to the isotype control. - <20%; + 20 to 40%; ++ 40 to 70%; +++ >70%.

Discussion

The development of effective therapies that specifically target critical components of the tumor signaling machinery requires the availability of significant numbers of malignant cells that accurately reflect the biology of the native cancer. This is particularly pertinent for the high-throughput screening of the increasing number of potentially active compounds made accessible by combinatorial chemistry libraries, computational chemistry and phage display libraries. To expedite the development of signaling-inhibitors active on T-cell leukemia and circumvent the limitations set by an insufficient number of primary leukemic cells and their generally poor *ex vivo* survival and viability, we have established an IL-7-dependent cell line – TAIL7 – from a pediatric patient. TAIL7 cells share the immunophenotype and clonality of their parental leukemic cells, as well as their responsiveness to IL-7 and IL-4. Importantly, stimulation of TAIL cells with IL-7 triggers different signaling events including the activation of the JAK/STAT pathway, hyperphosphorylation of Rb and downregulation of $p27^{kip1}$. Finally, specific blockade of JAK3 abrogates the proliferative and survival effect of IL-7 on TAIL7 cells, thus validating their use as a valuable tool for the screening of anti-T-ALL agents.

Tumor cell lines have been widely employed for several decades to test and develop anti-cancer drugs. However, significant problems with established cell lines have been reported, including multiple transformations and derivation, misidentification and cross-contamination with other cell line(s) [25]. The screening and validation of agents that inhibit signaling molecules or pathways in a specific manner especially requires cell lines that preserve the precise patterns of responsiveness to microenvironmental stimuli and the signaling pathways engaged by such stimuli. Most available cell lines derived from T-ALL patients have accumulated genetic lesions that may alter their phenotype, responsiveness to particular stimuli and, importantly, the integrity of the signaling pathways that are critical for their survival and growth. For example, 'classical' leukemic T-cell lines such as Jurkat, HPB-ALL, CEM, MOLT4, etc., are immortalized, growth factor-independent and often unresponsive to stimuli that impact on primary leukemic cells. This represents a marked disparity from primary leukemic cells, which undergo spontaneous apoptosis *ex vivo*. Moreover, the fact that the rate of primary T-ALL

apoptosis can be reduced by cytokines [19,26] or stromal cells [27], suggests that *in vivo*, microenvironmental signals are continuously rescuing the leukemic cells [28], a physiological need that the available T-ALL cell lines have overcome. Therefore, malignant T-cell lines that maintain the precise biological and molecular signature of their parental malignant cells are necessary for a more accurate comprehension of the biology of T-ALL, identification of therapeutic targets and design and development of more specific anti-leukemia drugs.

Several lines of evidence implicate IL-7 in the pathophysiology of lymphoblastic leukemia, particularly of T-cell ALL. First, leukemic T-cells express receptors for IL-7 (IL7-Rα and γc) and are responsive to IL-7, which promotes their survival and proliferation [4,9,10,19,29]. Second, IL-7 is present in the microenvironments where T-cell leukemia develops. In addition to the production of IL-7 by BM 'stromal' cells, we have also observed, in both normal donors and leukemia patients, that BM endothelial cells and mesenchymal stem cells express IL-7 (Yunes, Cardoso, et al., manuscript in preparation). Third, studies on IL-7 transgenic mice have shown that the deregulated expression of IL-7 perturbs thymic T-cell development and promotes malignant transformation of T and B lymphocytes [8]. Finally, IL-7 is involved in the development of normal T-cells, in particular of cells that represent the normal counterparts of malignant T-cells. It has been shown that IL-7 stimulates the growth of immature double negative and mature single positive thymocytes in thymic organ cultures [5,11,30,31]. In addition, stimulation of the IL-7R plays a critical role in the maintenance of the peripheral T-cell pool [6,32], and defective IL-7R expression results in $T^-B^+NK^+$ severe combined immunodeficiency [33,34]. Based on these multiple lines of evidence, we have selected IL-7 as the stimuli for the establishment of TAIL7 cells.

It is our contention that the dissection of the mechanisms involved in leukemic T-cell growth and survival in its "leukemia-favorable" microenvironments will lead to the identification of optimal targets for intervention and consequent development of more specific anti-leukemia drugs. An approach successfully employed in other tumors is to define the signaling pathways involved in the mediation of the critical stimuli implicated in tumor cell survival or growth [1,2,35,36]. We observed that in both TAIL7 cells and their parental leukemic T-cells, stimulation by IL-7 triggers the phosphorylation of various

substrates, and the activation of, at least two major cytokine-induced pathways, the JAK/STAT and the ras/MAPK pathway. Activation of JAK/STAT pathway has been shown in T-ALL cells and TEL-JAK2 transgenic mice develop T-cell leukemia [37-39]. The magnitude of the inhibitory effect of the JAK3 inhibitor WHI-P131 on TAIL7 cell survival and proliferation suggests that some degree of 'constitutive' activation of JAK3 occurs in TAIL7 cells, in addition to that mediated by the stimulation with IL-7. Activation of MAPK pathway has been implicated in thymic T-cell selection [40] and mutations that render $p21^{ras}$ constitutively active have been found in a wide variety of human tumors [35]. Consistent with our studies on primary leukemic T-cells [4], IL-7 stimulation of TAIL cells also modulates the expression and activation of cell cycle regulators as demonstrated by downregulation of $p27^{kip1}$ and hyperphosphorylation of Rb. This cell line can thereby be employed to screen agents that specifically inhibit critical steps on cell cycle progression and evaluate their efficacy as anti-leukemia agents. Finally, since TAIL7 cells express a defined pattern of T-cell oncogenes (high levels of *TAL1* and *LMO2,* and intermediate levels of *LYL1)* they can be a valuable tool to study transformation pathways in T-ALL and the role of misexpression of these transcription factors on tumor cell survival and proliferation.

In conclusion, we established a cell line derived from a T-ALL patient that preserves the biological responsiveness to IL-7 and the IL7-triggered engagement of defined signaling events seen for its parental primary leukemic cells. This novel, unique cell line is presently being used as a model system for the dissection of the signaling pathways implicated in T-ALL survival and growth, identification of therapeutic targets and, most importantly, the screening of putative anti-leukemia agents. Since TAIL7 accurately reflects the cellular and molecular properties of primary T-cell leukemia, it represents a valuable research tool.

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Chapter 5

ACTIVATION OF PI3K/AKT(PKB) PATHWAY IS INDISPENSABLE FOR IL-7-MEDIATED SURVIVAL AND CELL CYCLE PROGRESSION OF T-ALL CELLS

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In preparation

Abstract

Interleukin-7 (IL-7) is a cytokine critical for normal T-cell development and has been implicated in T-cell leukemogenesis. Previously, we have shown that IL-7 induces proliferation and increases viability of T-ALL cells by downregulating the cdk inhibitor $p27^{kip1}$, leading to cell cycle progression and Bcl-2 upregulation. Here, we sought to determine the signaling pathways via which IL-7 mediates these effects in T-ALL. To obtain adequate cell numbers to address these questions we utilized a cell line (TAIL7), established from the leukemic cells of patient with T-ALL, which displays proliferative and viability responses to IL-7 identical to primary leukemic cells. We investigated the role of p44/p42 MAPK (Erk1/2), $p38^{MAPK}$ and PI3K/Akt(PKB) pathways, which have active roles in normal T-cell expansion. IL-7 induced phosphorylation of Erk1/2, which was inhibited by the MEK inhibitor PD98059. However, inhibition of MEK/Erk pathway did not affect viability, $p27^{kip1}$ downregulation, Rb hyperphosphorylation and cell cycle progression, indicating that IL-7 mediated these events in a MEK/Erk-independent manner. In contrast to normal T-cells, in which the stress-activated $p38^{MAPK}$ pathway is activated by IL-7, $p38^{MAPK}$ remained unphosphorylated after IL-7 stimulation of TAIL7 cells. IL-7 induced PI3K-mediated phosphorylation of Akt and its downstream target GSK-3 in a time- and concentration-dependent manner. Specific inhibition of PI3K with LY294002 abrogated IL-7-mediated viability and cell cycle progression of both TAIL7 and primary T-ALL cells. Moreover, inhibition of PI3K blocked IL-7-mediated T-ALL activation, as assessed by cell size and expression of CD71 activation marker. LY294002 abrogated IL-7-mediated $p27^{kip1}$ downregulation and hyperphosphorylation of Rb and prevented upregulation of Bcl-2. These results implicate PI3K/Akt as a critical pathway for IL-7-induced viability, activation, and proliferation of T-ALL cells. Interestingly, although IL-7 induced phosphorylation of Akt and its downstream target GSK-3 it had no effect on FKHR, which was constitutively phosphorylated. This suggests that FKHR is inactivated in an Akt-independent manner and is not involved in IL-7-mediated viability and cell cycle progression. PTEN deficiency and PI3K/Akt overactivation are implicated in a variety of human cancers and were shown to be involved in tumorigenesis in mice. Our studies indicate that the PI3K/Akt/GSK-3 pathway appears to be involved in T-ALL cell expansion and may represent a novel molecular target for therapeutic intervention.

Introduction

IL-7 mediated signals are linked to survival and cell cycle progression [1]. In normal T-cell development, IL-7 plays a nonredundant role as an antiapoptotic factor by upregulating Bcl-2 expression [2]. Similar to normal immature thymocytes, leukemic blasts from T-cell acute lymphoblastic leukemia (T-ALL) patients can express functional IL-7 receptors [3,4]. Moreover, different studies demonstrated that IL-7 can induce proliferation and prevent spontaneous apoptosis of T-ALL cells *in vitro* [3,5,6], suggesting that IL-7 may play an important role in the biology of T-ALL. We have shown previously that IL-7 downregulates the expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$ thereby leading not only to cell cycle progression but also to upregulation of Bcl-2 protein expression and viability of T-ALL cells (Chapter 3; Ref. 7). We subsequently showed that IL-7 can support the long-term expansion of primary T-ALL cells, by establishing an IL-7-dependent cell line from the peripheral blood of a patient with T-ALL (chapter 4). This cell line - TAIL7 - possesses the essential features of IL-7-dependent primary T-ALL cells, and was used in this study to overcome the cell number limitations posed by primary leukemic cells.

In the present study we sought to determine the signaling pathways that mediate IL-7 effects on T-ALL cells. We investigated the role of p44/p42 MAPK (Erk1/2), $p38^{MAPK}$ and PI3K/Akt(PKB) pathways, which are involved in T-cell expansion [8-10].

We observed that IL-7 stimulation of T-ALL cells activates MEK/Erk pathway but fails to phosphorylate $p38^{MAPK}$. Experiments with the inhibitor PD98059 indicate that IL-7 promoted viability and proliferation in a MEK/Erk-independent manner. IL-7 also triggered phosphorylation of Akt and its downstream target GSK-3, which was critically dependent on PI3K activity. The PI3K inhibitor LY294002 abrogated cell survival and proliferation promoted by IL-7 on T-ALL cells. Our studies demonstrate that a PI3K pathway, probably involving Akt/PKB and GSK-3, is critical for IL-7-mediated T-ALL cell activation, proliferation and survival. PI3K/Akt(PKB) pathway may contribute to malignant T-cell expansion *in vivo* and represents a putative molecular target for pharmacological intervention in T-ALL.

Materials and Methods

Primary T-ALL samples and TAIL7 cell line. T-ALL cells were obtained from the peripheral blood and/or the bone marrow of patients with high leukemia involvement (85-100%). Informed consent and Institutional Review Board approval was obtained for all sample collections. Samples were enriched by density centrifugation over Ficoll-Hypaque and then washed twice in RPMI-1640 supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 2 mM L-glutamine (further referred to as RPMI-10 medium). The TAIL-7 cell line was established from the peripheral blood of a pediatric T-ALL patient and is responsive to IL-7 and IL-4 (Chapter 4).

***In vitro* culture**. Primary T-ALL or TAIL7 cells isolated by density centrifugation over Ficoll-Hypaque were cultured in 24-well plates as $2x10^6$ cells/ml at 37 C with 5% CO_2 in 1) RPMI-10 (control medium); 2) 10 ng/ml IL-7 (Endogen, Woburn, MA); 3) IL-7 plus 10μM MEK-specific inhibitor PD98059 (Calbiochem, San Diego, CA) or; 4) IL-7 plus 10μM PI-3K-specific inhibitor LY294002 (Calbiochem). At the indicated time points, cells were harvested and processed as indicated below for assessment of viability, activation, cell cycle progression and preparation of lysates for Western blotting. TAIL7 cells were previously starved in RPMI-10 without IL-7 for 5-7 days.

Proliferation assays. Cells were cultured in flat-bottom 96-well plates as $2x10^6$ cells/ml at 37°C with 5% CO2 in RPMI-10 without any cytokine or in the experimental conditions mentioned above. Cells were incubated with ^{3}H-Thymidine (1 μCi/well) for 16 hours prior to harvest. DNA synthesis, as measured by ^{3}H-Thymidine incorporation, was assessed using a liquid scintillation counter.

Assessment of cell viability and activation. Quantitative determination of viability of the malignant cells after culture under different conditions was performed by using an Annexin V-based apoptosis detection kit and the manufacturer's protocol (R&D Systems, Flanders, NJ). Briefly, cells were suspended in the appropriate binding buffer, stained

with FITC-conjugated Annexin V and propidium iodide at room temperature for 15 minutes, and subsequently analyzed by flow cytometry. Because activated cells have increased size and complexity, activation status was assessed by measuring changes in these physical parameters through analysis of SSC *versus* FSC flow cytometry plots gated on the live cell population. Surface expression of activation markers CD71 and CD69 was assessed by flow cytometry using FITC-conjugated anti-CD71 (Dako, Glostrup, Denmark) and PE-conjugated anti-CD69 (Beckman-Coulter, Fullerton, CA) and appropriately matched isotype controls. Irrelevant isotype-matched antibodies were used as negative controls. Samples were analyzed using a FACSCalibur flow cytometer and CELLQuest software (Becton-Dickinson, San Jose, CA). Results were expressed as the percentage of positive cells as compared to the negative control, and as the specific mean intensity of fluorescence (MIF), defined as the ratio of MIF of the specific antibody stain over the MIF of negative control antibody.

Cell cycle analysis. Determination of the percentage of cells at each stage of the cell cycle was performed by assessment of DNA content after staining with propidium iodide. Briefly, 5 x 10^5 cells per sample were resuspended in 0.5 ml PBS and then fixed with ice-cold 80% ethanol. Propidium iodide was added at a final concentration of 2.5 μg/ml, ribonuclease A was added at 50 μg/ml, and samples were incubated for 30 min at 37°C in the dark. Flow cytometric acquisition was performed using a FACSCalibur flow cytometer, and analysis of cell cycle histograms was carried out using ModFit LT software (Verity, Topsham, ME).

Intracellular signaling experiments. For the initial experiments, IL-7-deprived TAIL7 cells were washed twice with PBS and incubated for the indicated periods at 37°C with pre-warmed PBS alone or with the indicated concentrations of IL-7. Subsequently, IL-7-deprived TAIL7 cells were incubated for 15 minutes at 37°C with PBS alone or with 50 ng/ml of IL-7. In defined experiments, the cells were preincubated in PBS with 10μM LY294002, 10μM PD98059 or the corresponding volume of vehicle (DMSO) for 2 hours prior to stimulation. Reactions were stopped by placing samples on ice and adding ice-

cold PBS. Cells were washed twice with cold PBS and lysates were prepared for Western blot analysis (immunoblotting).

Immunoblotting, immunoprecipitation, and *in vitro* kinase reactions. Following the indicated conditions and time intervals of culture, cell lysates were prepared, and equal amounts of protein (50 μg/sample) were analyzed by 10% sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes, and immunoblotted with the indicated mAbs or antiserum: $p27^{kip1}$ (BD Transduction Laboratories, Lexington, KY), Actin, STAT5 and phospho-Erk1/2 (Y204) (Santa Cruz Biotechnology, Santa Cruz, CA), ZAP-70 and phospho-STAT5A/B (Y694/Y699) (Upstate Biotechnology, Lake placid, NY), phospho-Akt (S473), phospho-GSK-3β (S9), phospho-FKHR (S256), phospho-p38MAPK (T180/Y182), phospho-Erk1/2 (T202/Y204), Akt, p38MAPK and Erk1/2 (Cell Signaling Technology, Beverly, MA). To examine the phosphorylation status of Rb, proteins were analyzed by 6% SDS-PAGE, transferred onto nitrocellulose membrane, and blotted with Rb-specific mAb (PharMingen, San Diego, CA). After immunoblotting with mAbs or antiserum, immunodetection was performed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (1:5,000), anti-rabbit IgG (1:10,000) or anti-goat IgG (1:5,000) (Promega, Madison, WI) as indicated by the host origin of the primary antibody and developed by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Stripping and reprobing of the immunoblots were done as described [11].

Akt *in vitro* kinase reactions were performed using a non-radioactive Akt kinase assay kit purchased from Cell Signaling Technology and following the manufacturer's instructions. Briefly, cell lysates with equal amounts of protein were immunoprecipitated using agarose hydrazide-conjugated Akt antibody, washed twice and ressuspended in kinase buffer supplemented with 200μM cold ATP. Kinase reactions were performed using paramyosin-crosstide GSK-3α/β fusion protein as exogenous substrate. Reactions were analyzed by 12% SDS-PAGE, transferred to nitrocellulose membrane, and GSK-3 phosphorylation detected by immunoblotting with phospho-GSK-3α/β (Ser21/Ser9) antibody. Even loading was confirmed by stripping and reprobing the membranes with an Akt antibody (Cell Signaling Technology). Relative quantification of Western blot

bands was performed by densitometry analysis using ImageQuant Image Analysis software (Amersham Biosciences, Piscataway, NJ).

Intracellular staining. Bcl-2 protein expression was assessed at 72 and/or 96 hours of culture by intracellular staining. Cells were fixed in 0.1% formaldehyde for 30 minutes at 4°C, washed in PBS, resuspended in 1x Perm/Wash Solution (Pharmingen), and incubated with mouse monoclonal FITC-conjugated anti-Bcl-2 antibody (Dako). Irrelevant isotype-matched antibody was used as negative control. Propidium iodide was added to each sample in order to distinguish live from apoptotic cells. Samples were analyzed by flow cytometry. Results were expressed as the percentage of positive cells in comparison to the negative control, and as specific MIF.

Results

Akt, GSK-3 and Erk1/2, but not $p38^{MAPK}$, are phosphorylated by IL-7 in a time- and concentration-dependent manner

Phosphatidylinositol-3-kinase (PI3K)/ Akt pathway, as well as mitogen-activated protein kinase (MAPK) pathways MEK/ extracellular signal-regulated kinase (Erk) and $p38^{MAPK}$ were reported to play an active role in normal thymocyte and mature T-cell expansion [8-10,12,13]. The IL-7-dependent T-ALL cell line TAIL7 was used to investigate whether these pathways could be activated by IL-7 and to elucidate their functional role on IL-7-regulated T-ALL cell proliferation and viability. Cytokine-deprived TAIL7 cells were stimulated by IL-7 (50 ng/ml) for increasing periods of time for up to 120 minutes, and protein phosphorylation was assessed by Western blot. As seen in Figure 1A, phosphorylation of Akt at Ser473, necessary for Akt kinase full activation, occurred within 1 minute, was upregulated after 5 minutes and peaked at 30 minutes of stimulation with IL-7. After 1 hour, phosphorylated Akt was virtually absent. GSK-3, one of Akt downstream targets in several cell types, was phosphorylated with delayed but similar kinetics (Figure 1A), suggesting that Akt became enzymatically active after stimulation with IL-7. To confirm that IL-7 can activate Akt in TAIL7 cells, lysates from

unstimulated or 15 minute IL-7-stimulated cells were immunoprecipitated with anti-Akt antibody and an *in vitro* kinase reaction was performed using GSK-3α/β as exogenous substrate. Phosphorylation of GSK-3α and particularly GSK-3β was clearly upregulated (approximately 1.5- and 3-fold, respectively) by stimulation with IL-7, as shown in Figure 2. These results suggest that IL-7 induced Akt phosphorylation, leading to its activation and consequent phosphorylation of GSK-3.

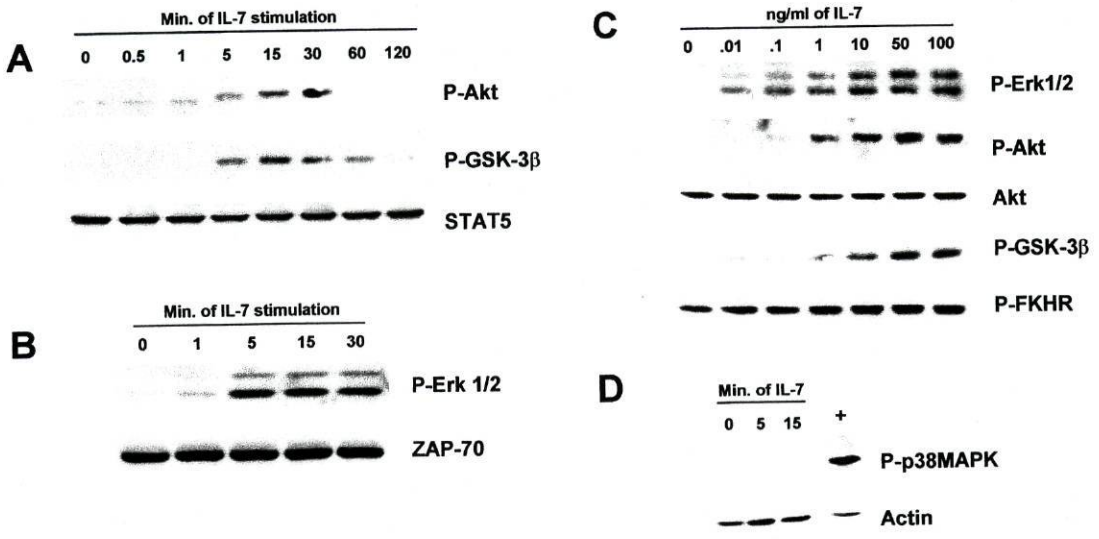

Figure 1. IL-7 phosphorylates Akt, GSK-3 and Erk1/2, but not p38^{MAPK}, in a time- and concentration-dependent manner. IL-7-deprived TAIL7 cells were stimulated with 50 ng/ml of IL-7 for the indicated periods **(A,B,D)**, or with increasing concentrations of IL-7 for 15 minutes **(C)**. Cell lysates were resolved with 10% SDS-PAGE and immunoblotted with the indicated antibodies. Results representative of at least three independent experiments are shown. Levels of phosphorylated Akt, GSK-3 and FKHR were analyzed by using antibodies that specifically recognize Ser473-phosphorylated Akt (P-Akt), Ser9-phosphorylated GSK-3β (P-GSK-3β) and Ser256-phosphorylated-FKHR (P-FKHR), respectively. Levels of phosphorylated Erk1/2 were detected with an antibody that reacts with Thr202/Tyr204-dual-phosphorylated Erk1 and Erk2 (P-Erk1/2). Same results were obtained with a Tyr204-phosphorylated-Erk1/2-specific antibody (data not shown). Blots were reprobed with anti-STAT5, ZAP-70, Akt or Actin antibodies to confirm even protein loading. Positive sign (+) in (D) indicates phospho-p38MAPK (Thr180/Tyr182) (P-p38MAPK) positive control lysate (IFN-γ-stimulated HeLa cells).

p44/p42 MAPK (Erk1/2) was phosphorylated and activated upon IL-7 stimulation of TAIL7 cells, as demonstrated by blotting with an antibody that detects Erk1/2 exclusively when catalytically activated by phosphorylation at both Thr202 and Tyr204. Phosphorylation of Erk1/2 was observed after 1 minute and reached a plateau between 5 to 15 minutes, after which it remained stable until at least 30 minutes of stimulation (Figure 1B). After 2 hours, phosphorylated Erk1/2 was still clearly detected (data not shown). Global levels of Akt and Erk1/2 remained unchanged (data not shown), indicating that variations in phosphorylation were not related to changes in protein expression. TAIL7 cells were then stimulated for 15 minutes with increasing doses of IL-7 and phosphorylation of Akt and Erk was determined. As shown in Figure 1C, phosphorylation of both molecules was detected at less than 1 ng/ml and reached a plateau at 10 ng/ml, with higher IL-7 concentrations not altering their phosphorylation levels.

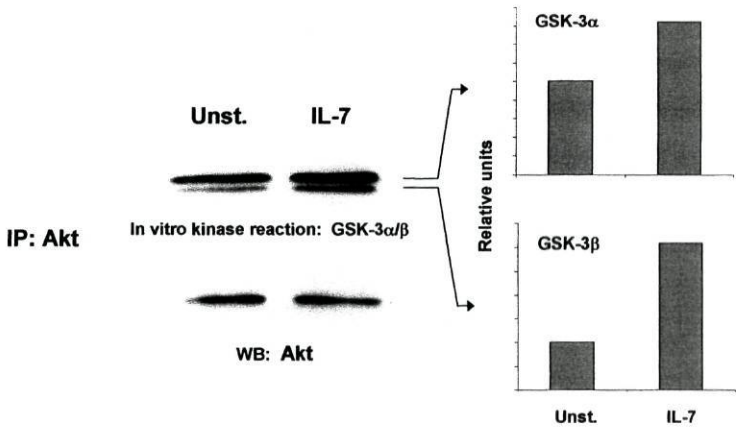

Figure 2. IL-7 activates Akt and induces *in vitro* phosphorylation of GSK-3 by Akt. IL-7-deprived TAIL7 cells were stimulated with 50 ng/ml of IL-7 for 15 minutes. To compare Akt enzymatic activity in unstimulated (Unst.) *versus* IL-7-stimulated cells (IL-7), cell lysates were immunoprecipitated with agarose-conjugated anti-Akt antibody and *in vitro* kinase reactions were done using crosstide-GSK-3α/β as exogenous substrate. Reactions were analyzed by 12% SDS-PAGE, transferred to nitrocellulose membrane, and GSK-3 phosphorylation detected by immunoblotting with anti-phospho-GSK-3α/β (Ser21/Ser9) antibody. Even loading was confirmed by stripping and reprobing the membranes with an anti-Akt antibody. Relative quantification of phosphorylated GSK-3α and GSK-3β bands was performed by densitometry analysis. Results were normalized in relation to the loading control (Akt) and expressed as relative units. IL-7 induced a 1.63-fold increase in GSK-3α and 3.07-fold increase in GSK-3β Akt-mediated phosphorylation. Results are representative of two independent experiments.

Members of the Forkhead family of transcription factors are direct targets of Akt [14,15]. Interestingly, FKHR was constitutively phosphorylated in unstimulated TAIL7 cells and we did not observe any significant changes in the phosphorylation level of FKHR in response to IL-7, even at high doses (Figure 1C). On the opposite, GSK-3 was phosphorylated in a dose-dependent fashion, in correlation with Akt activity. In contrast with Erk and Akt, the stress-activated $p38^{MAPK}$, which has been implicated in IL-7-induced T-cell proliferation [10], was not phosphorylated by IL-7 in TAIL7 cells (Figure 1D). Absence of phosphorylation indicated that p38MAPK was not activated by IL-7, since the antibody used recognizes p38 only when catalytically activated by dual phosphorylation at Thr180 and Tyr182. Thus, our results suggest that $p38^{MAPK}$ pathway is not involved in transducing IL-7 signals in T-ALL cells.

PI3K activity is necessary for IL-7-induced phosphorylation of Akt and GSK-3, whereas phosphorylation of Erk1/2 by IL-7 depends on MEK activity

IL-7 downstream pathways in T-cells are still relatively ill defined. Since we observed phosphorylation of Akt and GSK-3 mediated by IL-7, we shought to determine whether the PI3K/Akt pathway was functional in T-ALL cells. IL-7-deprived TAIL7 cells were pretreated with 10 μM of PI3K-specific inhibitor LY294002 for 2 hours prior to IL-7 stimulation. As shown in Figure 3A, LY294002 specifically abrogated phosphorylation of Akt and GSK-3, without affecting Erk1/2 or STAT5 phosphorylation. This suggests that IL-7-induced phosphorylation of Akt and consequently GSK-3 in T-ALL cells are absolutely dependent on PI3K activity. Moreover, our results indicate that activation of PI3K/Akt/GSK-3 pathway by IL-7 can be specifically disrupted by LY294002. FKHR constitutive phosphorylation was neither increased by IL-7 nor downregulated by LY294002 (Figure 3), indicating that this Forkhead-family member is not a downstream target for IL-7-activated PI3K/Akt pathway and it is not a mediator of IL-7 functional effects in TAIL7 cells.

IL-7 does not appear to phosphorylate Erk1/2 in normal T-cells [16]. To investigate whether IL-7-induced phosphorylation and activation of Erk1/2 in T-ALL cells was mediated by MEK, IL-7-deprived TAIL7 cells were pretreated with 10 μM of MEK-specific inhibitor PD98059 for 2 hours prior to IL-7 stimulation. As expected, PD98059

specifically inhibited phosphorylation of Erk1/2 at Thr202/Tyr204 without affecting Akt, GSK-3 or STAT5 phosphorylation (Figure 3B), indicating that Erk is activated in a MEK-dependent way upon stimulation of TAIL7 cells by IL-7 and that activation of MEK/Erk pathway by IL-7 is specifically inhibited by PD98059.

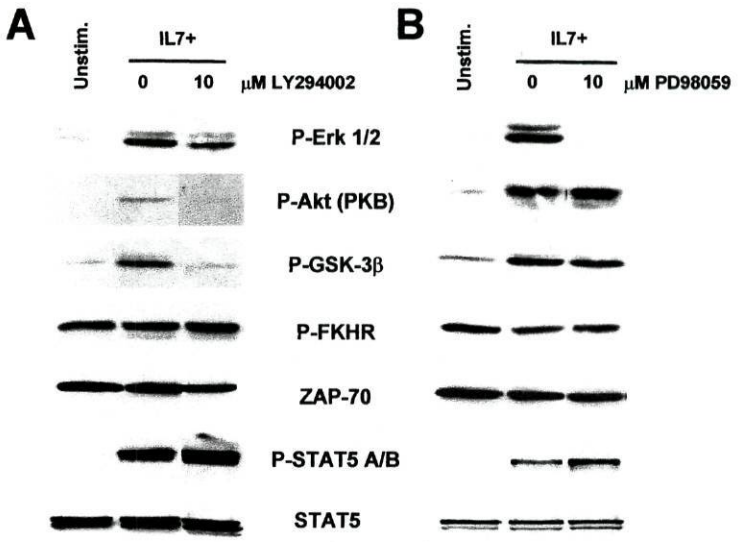

Figure 3. IL-7-induced phosphorylation of Erk1/2 and Akt/GSK-3 is specifically inhibited by PD98059 and LY294002, respectively. IL-7-deprived TAIL7 cells were pretreated with 10μM LY294002 **(A)** or 10μM PD098059 **(B)** for 2 hours and subsequently stimulated with 50 ng/ml of IL-7 for 15 minutes. Cell lysates were resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane and Western blot analysis was done with an antibody specific for phosphorylated Erk1/2. Blots were stripped and reprobed with the indicated antibodies. An antibody specific for Tyr694/Tyr699-phosphorylated-STAT5A/B was used to confirm that LY294002 and PD98059 were specific inhibitors of PI3K/Akt/GSK-3 pathway and MEK/Erk pathway, respectively. Anti-ZAP-70 and STAT5 antibodies were used to confirm equal loading. Representative results from three independent experiments.

PI-3K/Akt but not MEK/Erk pathway is required for IL-7-mediated increase in viability and cell cycle progression of T-ALL cells

To analyze the functional role of PI3K/Akt and MEK/Erk activation by IL-7 in T-ALL cells, we investigated the effect of specific inhibition of these pathways on viability and proliferation of IL-7-cultured TAIL7 cells, by co-culture with LY294002 and PD98059. Initial experiments determined that, consistent with primary T-ALL cells, IL-7 stimulated a dose-dependent increase in viability and proliferation of TAIL7 cells, which

was most evident after 72 and 96 hours of culture, with 10 ng/ml of IL-7 inducing maximal response (data not shown). Therefore, we used this concentration and time points in all subsequent experiments. Similar results were obtained at both 72 and 96 hours, although in general the differences between medium and IL-7 were more striking after 96 hours of culture. Staining of leukemic cells with annexin V-FITC and propidium iodide followed by flow cytometry analysis revealed that LY294002 inhibited IL-7-mediated increase in the percentage of viable cells, whereas PD98059 showed no effect (Figure 4A).

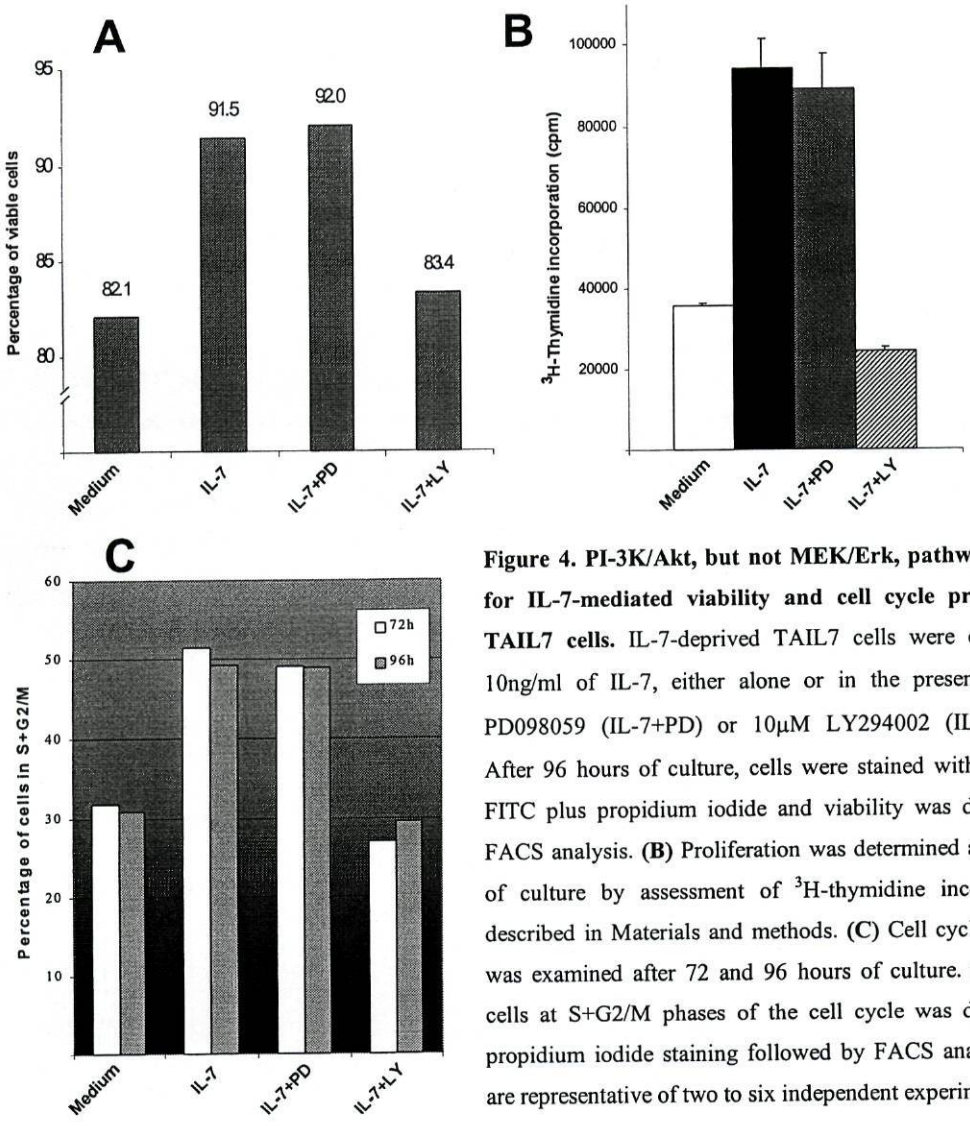

Figure 4. PI-3K/Akt, but not MEK/Erk, pathway is critical for IL-7-mediated viability and cell cycle progression of TAIL7 cells. IL-7-deprived TAIL7 cells were cultured with 10ng/ml of IL-7, either alone or in the presence of 10μM PD098059 (IL-7+PD) or 10μM LY294002 (IL-7+LY). **(A)** After 96 hours of culture, cells were stained with Annexin V-FITC plus propidium iodide and viability was determined by FACS analysis. **(B)** Proliferation was determined after 96 hours of culture by assessment of ^{3}H-thymidine incorporation as described in Materials and methods. **(C)** Cell cycle progression was examined after 72 and 96 hours of culture. Percentage of cells at S+G2/M phases of the cell cycle was determined by propidium iodide staining followed by FACS analysis. Results are representative of two to six independent experiments.

Similar results were obtained when we examined synthesis of DNA as a measure of cell proliferation, by assessing the cellular incorporation of ^{3}H-thymidine. As shown in Figure 4B, PD98059 did not affect proliferation of IL-7-cultured TAIL7 cells. In contrast, LY294002 completely abrogated IL-7-induced proliferation. To confirm whether inhibition of PI3K/Akt pathway blocked proliferation not only because it impaired viability but also because it actually downregulated cell cycle progression, TAIL7 cells cultured for 72 and 96 hours were fixed and stained with propidium iodide for flow cytometry cell cycle analysis. As shown in Figure 4C, LY294002 completely inhibited cell cycle progression of IL-7-cultured cells, while as expected PD98059 had no effect.

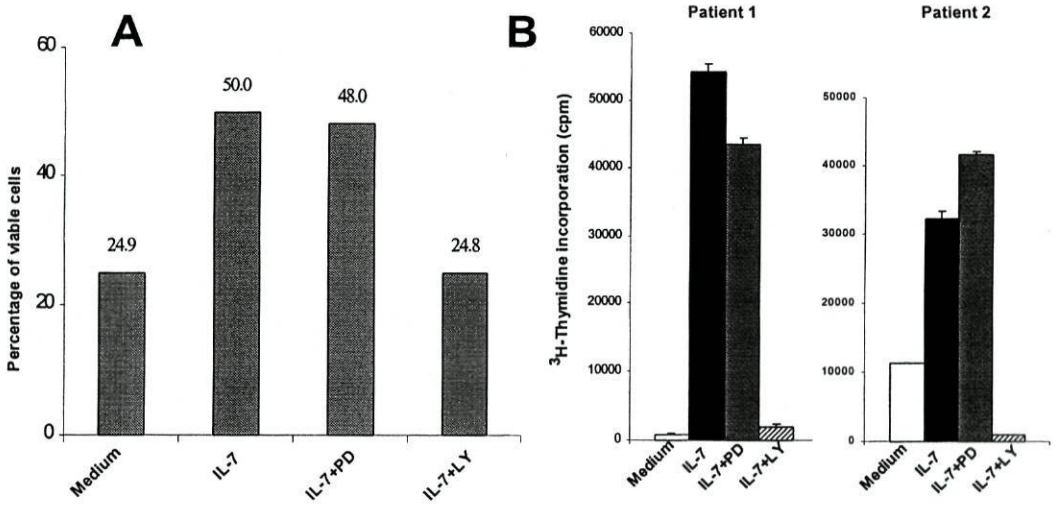

Figure 5. PI-3K/Akt, but not MEK/Erk, pathway is critical for IL-7-mediated increase in viability and cell cycle progression of primary T-ALL cells. Primary T-ALL cells were cultured with 10ng/ml of IL-7, either alone or in the presence of 10μM PD098059 (IL-7+PD) or 10μM LY294002 (IL-7+LY). **(A)** After 96 hours of culture cells were stained with Annexin V-FITC and propidium iodide. Percentage of viable cells was determined by FACS analysis. **(B)** Proliferation was determined after 72 hours of culture by assessment of ^{3}H-thymidine incorporation as described in Materials and methods. Results are representative of two independent experiments with three primary T-ALL samples.

To extend the biological significance of these results, we examined whether PI3K/Akt and MEK/Erk pathways played similar functional roles in primary T-ALL cells in response to IL-7. Highly enriched leukemic cells were collected from the peripheral blood or bone marrow of pediatric patients with T-ALL and tested for *in vitro*

responsiveness to IL-7, as assessed by increased proliferation when cultured with the cytokine. IL-7-responsive samples were selected for subsequent experiments. As shown in Figure 5A for one representative case, the increase in viability observed after 96 hours of culture with IL-7 was not affected by addition of PD98059, whereas LY294002 completely blocked IL-7-induced T-ALL cell survival. Proliferation in response to IL-7 was also abrogated by LY294002 (Figure 5B). In contrast, PD98059 either slightly upregulated or did not significantly downregulate IL-7-mediated proliferation of primary T-ALL cells. Results from TAIL7 and primary T-ALL cells demonstrate that PI3K/Akt pathway is indispensable for both viability and cell cycle progression of IL-7-stimulated T-ALL cells. In contrast, MEK/Erk pathway albeit activated by IL-7 appears not to be functionally involved in mediating either of these effects in T-ALL cells.

IL-7-mediated downregulation of $p27^{kip1}$, Rb hyperphosphorylation and upregulation of Bcl-2 in T-ALL cells is dependent upon PI-3K/Akt activity

Cell cycle progression from G0/G1 to S phase is modulated by exogenous factors and is positively regulated by cyclin/cyclin-dependent kinase (cdk) holoenzymes, which phosphorylate several intracellular substrates including Rb [17]. Phosphorylation and consequent inactivation of Rb results in release of E2F transcription factors thereby allowing transcription of their target genes, which are required for progression through S phase [18,19]. The enzymatic activity of cyclin/cdk complexes is negatively regulated by cdk inhibitors, including the cip/kip-family member $p27^{kip1}$ [20,21]. We previously demonstrated that culture with IL-7 downregulates $p27^{kip1}$ protein expression resulting in cdk activation, ensuing hyperphosphorylation of Rb and cell cycle progression [7]. Because LY294002 inhibited IL-7-regulated cell cycle progression, we next evaluated whether PI3K/Akt pathway could indeed link IL-7 to the cell cycle machinery. TAIL7 cells cultured with IL-7 for 96 hours downregulated the expression of $p27^{kip1}$ (Figure 6A) and showed hyperphosphorylation of Rb (Figure 6B). LY294002 completely reversed both IL-7-mediated effects, upregulating $p27^{kip1}$ to the same levels as those presented by leukemic cells cultured in medium alone, and preventing Rb phosphorylation. PD98059 was used to confirm that these effects were specific. As expected, the MEK/Erk pathway

inhibitor had no effect. These results strongly suggest that IL-7 induces $p27^{kip1}$ downregulation and cell cycle progression via PI3K/Akt pathway.

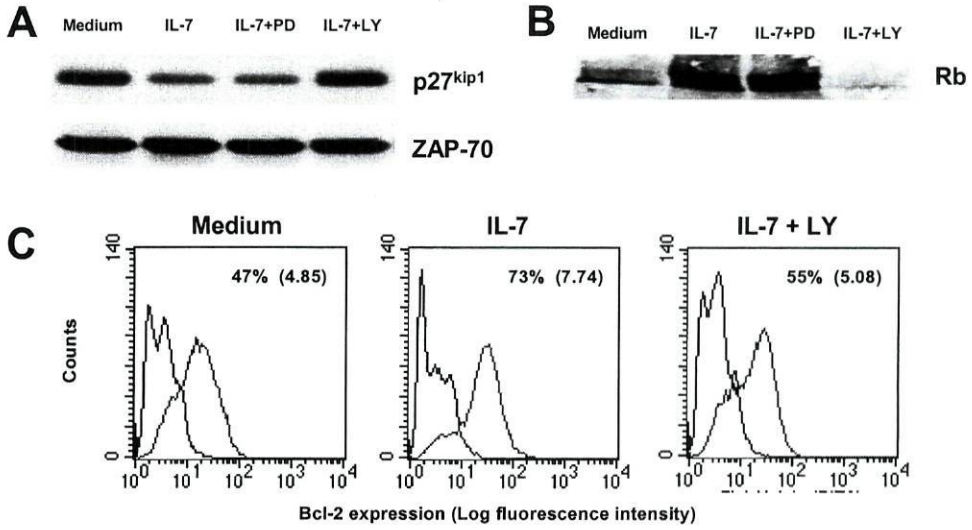

Figure 6. IL-7 mediates $p27^{kip1}$ downregulation, Rb hyperphosphorylation and Bcl-2 upregulation via activation of PI-3K/Akt pathway in T-ALL cells. IL-7-deprived TAIL7 cells were cultured for 96 hours under the indicated conditions. **(A)** Cell lysates were resolved by 10% SDS-PAGE and immunoblotted with anti-$p27^{kip1}$ antibody. Membranes were stripped and reprobed with ZAP-70 to confirm equal loading. **(B)** Lysates from the same samples were analyzed by 6% SDS-PAGE and immunobloted with an Rb-specific antibody. Hyperphosphorylated form of Rb corresponds to the band with the higher apparent molecular weight. **(C)** Bcl-2 protein levels were assessed by FACS analysis after intracellular staining of fixed and permeabilized cells with FITC-conjugated anti-Bcl-2 antibody. Results were expressed as the percentage of positive cells as compared to the negative control, and as the specific mean intensity of fluorescence (MIF), defined as the ratio of MIF of the Bcl-2-specific antibody stain over the MIF of negative control antibody (in brackets in the respective histogram). Results represent at least three independent experiments.

Prevention of spontaneous apoptosis of *in vitro*-cultured primary T-ALL cells by IL-7 was shown to correlate with increased Bcl-2 protein expression [6]. We later demonstrated that Bcl-2 upregulation was mandatory for IL-7-mediated promotion of viability and that $p27^{kip1}$ downregulation was at least partially responsible for increased expression of Bcl-2 [7]. We therefore examined whether IL-7 regulated Bcl-2 via activation

of PI3K. TAIL7 cells were cultured in the absence of cytokine, with IL-7 or with IL-7 plus LY294002. After 96 hours, cells were fixed, stained intracellularly with anti-Bcl-2 antibody and analyzed by flow cytometry. IL-7-mediated upregulation of Bcl-2 was significantly impaired by LY294002, as determined by the decrease in the percentage of Bcl-2 positive cells and in the specific mean intensity of fluorescence (Figure 6C). This suggests that IL-7 upregulates Bcl-2, at least in part, by activating PI3K/Akt pathway in T-ALL cells.

IL-7 induces T-ALL cell activation, as assessed by increased cell size and expression of CD69 and CD71 activation markers

Proliferation of T lymphocytes is normally associated with cellular activation, which is measured by the expression of certain surface antigens such as CD69 and CD71. Activated T-cells increase their size and cellular complexity and therefore can also be identified by analysis of these morphological parameters. We investigated whether the proliferative effect of IL-7 on T-ALL cells could likewise be associated with T-ALL cell activation and not solely with cell cycle progression and cell division. Primary T-ALL cells were cultured with IL-7. After 72 hours of culture, cells were harvested and immediately evaluated for size and complexity by flow cytometry. As depicted in Figure 7A for one representative patient, FSC *versus* SSC plots gated on the live cell population showed that IL-7 increased both cell size and intracellular complexity of primary T-ALL cells (9 of 9 cases analyzed). Similar results were obtained using TAIL7 cell line (Figure 7B), as we had previously observed (Chapter 4). Hence, we used this T-ALL cell line to compare the surface expression of activation markers CD71 and CD69 between medium- and IL-7- cultured cells. Flow cytometry analysis revealed that both CD69 and CD71 were strongly upregulated by IL-7, although the increase in CD71 surface expression was more dramatic (Figure 7C). Therefore, IL-7 was capable of inducing some of the morphological and immunophenotypical changes in T-ALL cells characteristic of activated T lymphocytes.

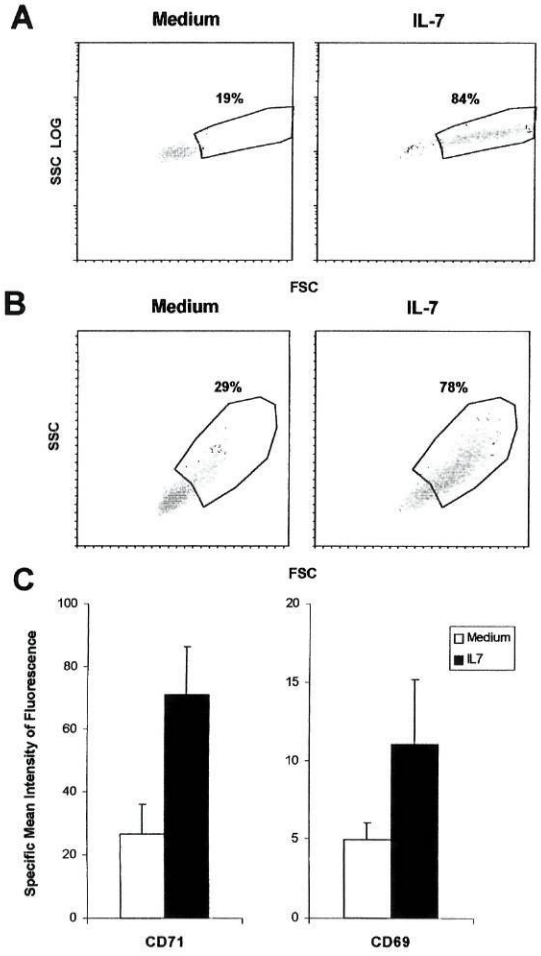

Figure 7. IL-7 induces activation of TAIL7 and primary T-ALL cells. Primary T-ALL cells **(A)** and TAIL7 cell line **(B)** were cultured for 72 hours in medium alone or with IL-7 (10 ng/ml) and then analyzed by FACS for changes in cell size (determined by FSC) and intracellular complexity (determined by SSC) in the live cell population. Percentage of activated cells was calculated by defining a gate that excluded the bulk, small-sized population of medium-cultured cells, thereby allowing relative comparisons. **(C)** TAIL7 cell line was cultured for 96 hours under the same conditions and expression of CD71 and CD69 surface antigens was examined by FACS. Results were expressed as the specific mean intensity of fluorescence (MIF), as defined in Materials and methods, and represent the average and standard deviation from three independent experiments. Results from one representative patient of nine tested are shown in (B). Results shown in (A) are similar to those found in six independent experiments.

Activation of T-ALL cells mediated by IL-7 is impaired by inhibition of PI3K/Akt pathway

To determine which intracellular pathways were involved in mediating IL-7-induced activation of T-ALL cells, we blocked IL-7-activated PI3K/Akt and MEK/Erk pathways with the respective specific inhibitors. After 96 hours of culture, TAIL7 and primary T-ALL cells were analyzed for changes in cell size and complexity. LY294002 completely inhibited or greatly impaired activation of IL-7-cultured TAIL7 and primary T-ALL cells (Figure 8A,B and data not shown). In contrast, PD98059 originated only a minor decrease in the percentage of activated cells. To confirm the importance of PI3K/Akt in IL-7-mediated T-ALL cell activation we next analyzed the expression of

CD71, the activation marker whose intensity of expression was more dramatically upregulated by IL-7. As shown in Figure 8C, LY294002 completely inhibited IL-7-mediated surface expression of CD71 in TAIL7 cells, as assessed by both specific mean intensity of fluorescence and percentage of CD71-positive cells. Blockade of MEK/Erk pathway by PD98002 induced a partial reduction in the intensity of expression of CD71 in IL-7-cultured cells, although the percentage of CD71-positive cells remained virtually unaltered. These results imply that PI3K/Akt pathway, in contrast to MEK/Erk pathway, plays a major role in IL-7-mediated activation of both TAIL7 and primary T-ALL cells.

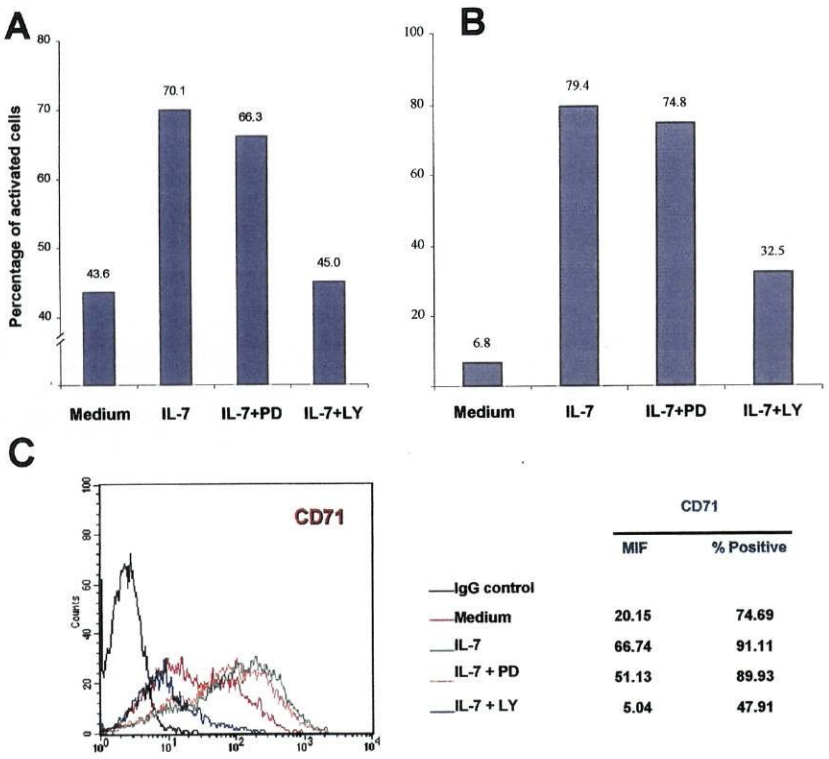

Figure 8. PI-3K/Akt but not MEK/Erk pathway is critical for IL-7-mediated activation of T-ALL cells. IL-7-deprived TAIL7 cells **(A)** and primary T-ALL cells **(B)** were cultured for 96 hours under the indicated conditions, and percentage of activated cells was calculated by FACS analysis of FSC x SSC plots gated on the live cell population. **(C)** TAIL7 cells cultured for 96 hours under the indicated conditions were stained with anti-CD71 antibody. Results from FACS analysis were expressed as the percentage of positive cells as compared to the negative control, and as the specific mean intensity of fluorescence (MIF), as defined in Materials and methods. The overlay histogram represents the staining of CD71 under the different conditions and the IgG control corresponds to the negative control stain from IL-7. The remaining conditions presented a similar IgG control stain (data not shown). Results from primary T-ALL cells are from one representative patient of three patients tested. Results from TAIL7 cell line were similar in three independent experiments.

Discussion

IL-7 is expressed in the bone marrow and thymus and has been shown to stimulate the expansion of immature double negative and mature single positive thymocytes in part by upregulating Bcl-2 expression and viability [1,22], but also by inducing cell cycle progression [1,23]. In humans and mice, defective IL-7R expression results in severe T-cell deficiency [24-26], indicating that IL-7 plays an essential role during T-cell ontogeny. Primary leukemic T-cells clearly show increased proliferation [3,5] and viability [6] when cultured with IL-7, suggesting that IL-7 might also be involved in the biology of T-ALL. We have previously identified the cdk inhibitor $p27^{kip1}$ as a critical molecule for IL-7-mediated effects in T-ALL [7]. Upregulation of cdk activity with consequent Rb hyperphosphorylation and progression towards S-phase are absolutely dependent upon IL-7-induced downregulation of $p27^{kip1}$. In addition, we found that $p27^{kip1}$ downregulation is associated with upregulation of Bcl-2, which in turn is essential for IL-7-mediated survival of T-ALL cells.

In the present study, we examined some of the signaling pathways that could link IL-7 to the downstream apoptosis and cell cycle regulators, particularly to $p27^{kip1}$ and Bcl-2, thereby modulating the proliferation and viability of T-ALL cells. Knowledge regarding IL-7-mediated pathways in T-cells is rather incomplete (reviewed in Chapter 1 and in Ref. [16]) and nothing is known about the integrity and biological role of those pathways in T-ALL cells. PI3K/Akt, MEK/Erk and p38MAPK pathways have been associated with TCR- or cytokine-promoted expansion of T-cells and T-cell precursors [12,13]. However, only PI3K/Akt and p38MAPK appear to be activated by IL-7 in normal T-cells [9,10,27]. Several groups studied the possibility of IL-7 activating MEK/Erk pathway. Only one group reported mild phosphorylation of p44MAPK (Erk1) by IL-7 in a murine T helper cell clone [28], whereas the remaining studies found no evidence of Erk1/2 phosphorylation [10,27,29]. Here, we demonstrate that in T-ALL cells, IL-7 clearly activates Erk1 and Erk2 in a time- and dose-dependent manner and relying on MEK activity, since the MEK-specific inhibitor PD98059 completely and specifically abrogates Erk activation. Importantly, inhibition of MEK/Erk pathway does not affect IL-7-mediated viability or cell cycle progression, indicating that these events occur in a MEK/Erk-

independent manner. Other studies support a critical role for Ras and MEK/Erk in $p27^{kip1}$ phosphorylation and consequent degradation by the ubiquitin-proteasome system [30]. However, we found that downregulation of $p27^{kip1}$ protein expression or Rb hyperphosphorylation that results from culture with IL-7 cannot not be reverted by MEK inhibition in TAIL7 cells. Hence, although MEK/Erk pathway is activated by IL-7 in T-ALL cells, its exact biological role in IL-7-related signaling remains to be determined.

In normal T-cells, the stress-activated p38 MAPK pathway is activated by IL-7 and is required for IL-7-mediated proliferation [10]. In contrast, we show that p38 is not activated by IL-7 in T-ALL cells. Activation of p38 MAPK has been implicated in thymocyte apoptosis and negative selection [31,32]. Thus, despite being involved in T-cell proliferation, p38 can also induce cell death. Because IL-7 has the capacity to mediate T-ALL cell proliferation via p38 MAPK-independent mechanisms and, therefore, does not activate a potentially proapoptotic pathway, we hypothesize that this confers a selective advantage to T-ALL cells over their normal counterparts.

Our present study shows that IL-7 induces phosphorylation of Akt and GSK-3 in a PI3K-dependent manner, indicating the existence of a functional IL-7-mediated PI3K/Akt pathway in T-ALL cells. Activation of this pathway is mandatory for $p27^{kip1}$ downregulation, Rb hyperphosphorylation and Bcl-2 upregulation in IL-7-cultured T-ALL cells. Accordingly, IL-7 mediates cell cycle progression and viability of T-ALL cells via PI3K/Akt pathway. FKHR is constitutively phosphorylated in unstimulated TAIL7 cells and we did not observe any significant changes in the Ser256 phosphorylation level of FKHR after IL-7 stimulation or when cells were pretreated with LY294002 before IL-7 stimulation (data not shown). These results suggest that FKHR plays no role in IL-7-mediated effects in TAIL7 cell line and raise the question of whether FKHR constitutive phosphorylation is relevant for the progression of the disease. In future studies, it will be interesting to examine whether primary T-ALL cells also express constitutively phosphorylated FKHR, and if so, to elucidate the mechanism for this constitutive phosphorylation which, remarkably, appears to be independent of PI3K/Akt activation. TAIL7 cells express the tumor suppressor PTEN (data not shown), a lipid phosphatase that hydrolyses 3-phosphoinositides thereby counteracting the action of PI3K. Thus, constitutive phosphorylation of FKHR cannot result from overactivation of

PI3K or Akt due to PTEN deficiency. Finally, it should be stressed that FKHRL1 and AFX were not analyzed and hence one cannot exclude the possibility that these Forkhead transcription factors can be relevant targets for IL-7-activated PI3K/Akt pathway. However, expression of the Forkhead target FasL was hardly affected by culture with IL-7 or IL-7 plus LY294002 (data not shown), suggesting two possibilities: either FKHRL1 and AFX, as FKHR, are not targets for IL-7-mediated pathways or FasL is not regulated by these transcription factors in T-ALL cells. In any case, IL-7 appears not to regulate FasL expression in T-ALL cells.

Numerous studies have shown that engagement of the IL-7 receptor induces activation of PI-3K and PI(3,4,5)P3 production in human thymocytes, T-lineage ALL blasts and T-ALL cell lines [33-36], leading to their proliferation and survival [9,36]. However, the exact PI3K-dependent mechanisms through which IL-7 exerts its effects in T-cells are still being explored. PI3K/Akt pathway mediates viability and cell cycle progression in a variety of cell types [37]. Both effects appear to be accomplished via Akt direct inhibition of several downstream targets. First, Akt phosphorylates caspase 9 at Ser196 and Bad at Ser136, leading to their inactivation [38,39]. Second, phosphorylation of Forkhead family members FKHR, FKHRL1 and AFX by Akt induces their inactivation and nuclear export [14,15]. Third, FasL and $p27^{kip1}$, which can be involved in mediating apoptosis, are transcriptionally upregulated by Forkheads [15,40], with $p27^{kip1}$ acting as a critical negative regulator of cell cycle progression [17,20,21]. Fourth, Akt inhibition of GSK-3α and GSK-3β by phosphorylation at Ser21 and Ser9, respectively, can also lead to increased viability, since GSK-3 mediates cell death by mechanisms that are still unidentified [41,42]. In addition, GSK-3β was shown to phosphorylate cyclin D1 [43] and c-Myc [44], promoting their protein degradation and thus contributing to cell cycle arrest. Moreover, nuclear factor of activated T-cells (NF-ATc), another transcription factor involved in proliferation, is also phosphorylated by GSK-3, resulting in enhanced nuclear export and inhibition of NF-ATc ability to bind DNA [45,46]. NF-ATc also appears to mediate survival by regulating Bcl-2 gene transcription [47]. Therefore, IL-7-mediated activation of PI3K/Akt pathway may lead to T-ALL cell cycle progression and protection from apoptosis by inactivating GSK-3. In this study, we show that PI3K-dependent activation of Akt by IL-7 mediates GSK-3α/β *in vitro* phosphorylation, suggesting that the same

effect is exerted by Akt *in vivo*. GSK-3 phosphorylation and subsequent inactivation could result in Bcl-2 upregulation via activation of NF-ATc transcriptional activity [47], and $p27^{kip1}$ downregulation via c-Myc protein stabilization [44,48]. Interestingly, Akt was recently shown to be able to directly phosphorylate $p27^{kip1}$ at Ser10, Thr187 and a newly identified target residue, Thr198, therefore promoting $p27^{kip1}$ binding to 14-3-3 proteins, cytoplasmic localization and degradation by the ubiquitin-proteasome pathway [49]. Thus, it is possible that Akt may also directly phosphorylate $p27^{kip1}$ and induce its protein downregulation in IL-7-stimulated T-ALL cells. In turn, $p27^{kip1}$ downregulation would contribute to Bcl-2 upregulation by as yet unknown mechanisms [7].

By definition leukemic T-cells are lymphoblasts, i.e. activated lymphoid cells. However, we found that IL-7 has the capacity to further activate T-ALL cells, leading to a dramatic increase in cell size and surface expression of the activation markers CD71 and CD69. Whereas PD98059 does not significantly affect IL-7-mediated activation of T-ALL cells, LY294002 clearly downregulates cell size and completely abrogates expression of CD71 in IL-7-cultured T-ALL cells. Our results indicate that PI3K/Akt pathway plays a fundamental role in IL-7-mediated activation of T-ALL cells.

IL-7 binding to its receptor directly activates Jak1 and Jak3 kinases and their substrates of the STAT family of transcription factors, most notably STAT5 [16]. IL-7-mediated activation of STAT5 induces differentiation of human thymocyte precursors [9]. Although we have shown (Chapter 4 and Supplementary Data, this Chapter) that IL-7 induces phosphorylation and DNA-binding activity of STATs in TAIL7 cells, in this study we did not analyze the functional role of Jak/STAT pathway. Engagement of IL-2 receptor promotes T-cell proliferation and induction of genes involved in proliferation (c-Myc) and survival (Bcl-2 and Bcl-x) through the transcriptional activation domain of STAT5 [50]. In addition, STATs can induce downregulation of $p27^{kip1}$, which correlates with the ability of cytokines to mediate both cell cycle progression and malignant transformation [51]. Interestingly, Jak/STAT pathway has been shown to act synergistically with other pathways, including PI3K/Akt, to maximize cytokine-dependent cell growth and survival [52,53]. Therefore, further studies are required to elucidate whether Jak/STAT pathway might collaborate with PI3K/Akt in IL-7-associated cell cycle progression and viability of T-ALL cells.

Regardless of the fact that synergistic effects with other pathways were not investigated, the results presented here demonstrate that the PI3K/Akt pathway is fundamental for all the effects related to the role of IL-7 in T-ALL cell biology. Activation, proliferation and survival of T-ALL cells are mediated by IL-7 in a PI3K-dependent manner. Accordingly, downregulation of $p27^{kip1}$ and increased expression of Bcl-2 depend upon activation of PI3K/Akt. Overactivation of PI3K/Akt pathway is implicated in a variety of human cancers and is associated with tumorigenesis in mice [54-56]. Mice heterozygous for PTEN-inactivating mutations have a high tendency to develop malignancies of various origins, including T-cell lymphomas [57], whereas mice with T-cell-specific deletion of PTEN die from the occurrence of T-cell lymphomas [58]. Moreover, in human hematological malignancies there is an inverse correlation between expression levels of PTEN and phosphorylated Akt [59]. Strikingly, Jurkat and other T-ALL cell lines lack expression of PTEN and consequently have high PI3K and Akt constitutive activities [60,61]. These studies reveal the importance of a tight regulation of PI3K/Akt pathway for normal development, activation and cell death, and underscore the consequences of breaking that control for the progression of cancer. In view of these facts and our own results, we suggest that PI3K/Akt(PKB) pathway may contribute to T-ALL cell expansion *in vivo* and, therefore, may serve as an optimal molecular target for pharmacological intervention in this malignancy.

Acknowledgements

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SUPPLEMENTARY DATA

IL-7 activates Jak/STAT pathway in T-ALL cells

We evaluated the activation of Jak/STAT pathway in response to IL-7. Stimulation of TAIL7 cells with IL-7 clearly induced tyrosine phosphorylation of STAT5a/b in a time- and concentration-dependent manner (Figure 9A,B). In accordance, IL-7 mediated the activation of STAT5, as assessed by increased DNA-binding activity (Figure 9C). IL-7 was also capable of inducing tyrosine phosphorylation of STAT1 and STAT3 (Figure 9D). Although we previously detected some degree of Jak3 basal activation in unstimulated TAIL7 cells (Chapter 4), no constitutive phosphorylation of STAT1, STAT3 or STAT5 was observed in this cell line (Figure 9A-D). Likewise, the factor-independent T-ALL cell line Jurkat (data not shown), as well as primary T-ALL cells (Figure 9E,F) did not show STAT5 constitutive phosphorylation. Lack of STAT5 constitutive phosphorylation was not the consequence of absence of STAT5 protein expression, as seen in Figure 9F and 9G.

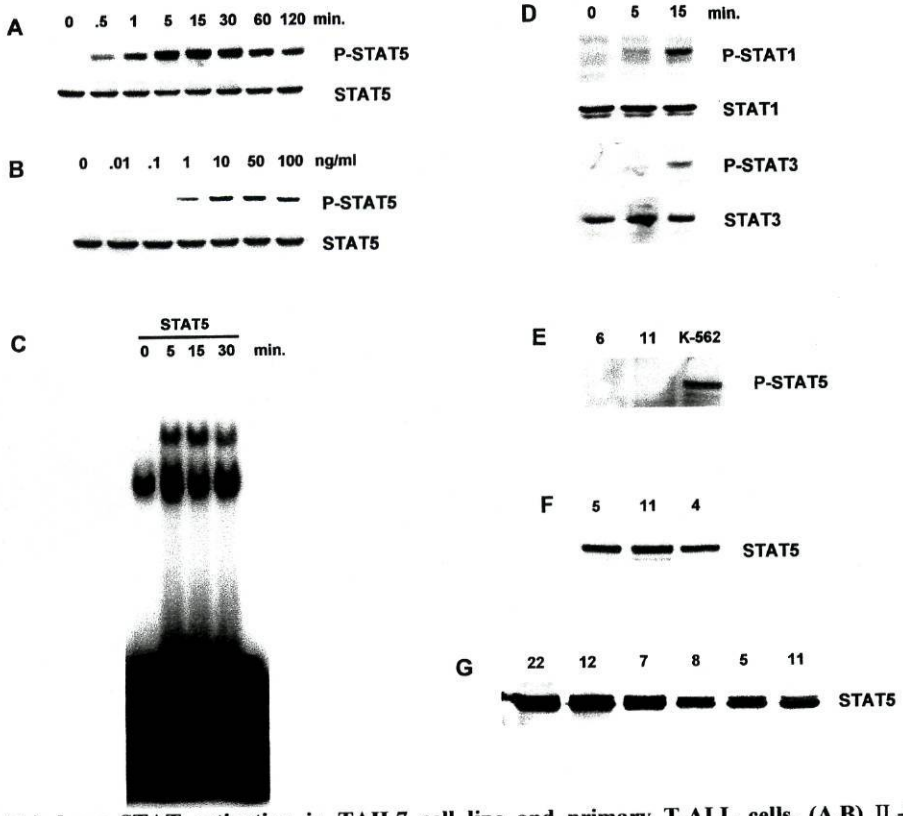

Figure 9. IL-7 induces STAT activation in TAIL7 cell line and primary T-ALL cells. (A,B) IL-7 induces STAT5a/b tyrosine phosphorylation in a time- and concentration-dependent manner. TAIL7 cells were starved and then stimulated with 50ng/ml of IL-7 for the indicated time points **(A)** or were stimulated for 15 minutes with the indicated concentrations of IL-7 **(B)**, lysates were resolved by SDS-PAGE and tyrosine phosphorylation of STAT5 was determined by western blot. **(C)** IL-7 induces STAT5 DNA-binding activity. Nuclear extracts were prepared from unstimulated (0 minutes) and IL-7-stimulated (5, 15 and 30 minutes) TAIL7 cells. EMSA was performed with a STAT5 consensus oligonucleotide. **(D)** TAIL7 cells were stimulated with IL-7 for the indicated time points and tyrosine phosphorylation of STAT1 and STAT3 was determined by western blot. **(E)** Lysates from primary T-ALL cells were probed for STAT5 tyrosine phosphorylation by western blot. K-562 erythroleukemia cell line was used as a positive control. Results representative of 2 out of 5 patients tested. T-ALL sample number is indicated above the corresponding lane. **(F,G)** Primary T-ALL cells were analyzed for STAT5 protein expression. T-ALL sample number is indicated above the corresponding lane.

Materials and Methods

Immunoblotting. Following the indicated conditions and time intervals of stimulation, cell lysates were prepared, and normalized amounts of protein (50 μg/sample) were analyzed by 10% sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes, and immunoblotted with the indicated monoclonal antibodies (mAbs) or antiserum. STAT1 p84/p91 and STAT5 antisera, and STAT3 mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-STAT1 (Y701) antiserum, phospho-STAT3 (Y704), phospho-STAT5a/b (Y694/Y699) and ZAP-70 mAbs were purchased from Upstate Biotechnology (Lake Placid, NY). After immunoblotting with mAbs or antiserum, immunodetection was performed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (1:5,000) or anti-rabbit IgG (1:10,000) (Promega, Madison, WI) as indicated by the host origin of the primary antibody and developed by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were stripped and reprobed when necessary. Briefly, membranes were incubated at 56°C for 30 minutes with stripping buffer (Tris-HCl pH 6.7, 2-Mercaptoethanol 100mM, Sodium Dodecyl Sulfate (SDS) 2%), washed and reused for analysis of other proteins.

Nuclear extracts and electrophoretic mobility shift assay. Nuclear extracts from unstimulated or IL-7-stimulated T-ALL cells were prepared as follows: cells were harvested and washed twice in PBS, the cell pellet was ressuspended in extraction buffer (Hepes 20 mM pH 7.8, NaCl 450 mM, EDTA 10 mM, DTT 0.5 mM, Glycerol 25% vol/vol, PMSF 0.5 mM), and then freeze-thawed 3 times, spun down for 10 minutes at 14,000 rpm and the supernatant collected. Protein concentration was determined and 10 μg per sample were used for electrophoretic mobility shift assays (EMSAs). EMSA were performed using STAT5 gel shift oligonucleotides purchased from Santa Cruz Biotechnology and following standard methodology. Briefly, STAT5 consensus oligonucleotide probes were labeled with γ^{32}-ATP, and radioactivity was measured using a scintillation counter. Labeled probe (20,000 cpm) was added to 10 μg of protein in reaction buffer (20 mM Hepes, 1 mM $MgCl_2$, 0.5 mM DTT and poly(dI-dC) 4%, BSA 24% and glycerol 20%). Binding reaction mixtures were incubated at 30°C for 30 minutes. DNA-protein complexes were resolved by non-denaturing 4% polyacrylamide gel electrophoresis. The gels were subsequently dried and autoradiographed at -70°C for as long as necessary. Binding reaction mixtures without any protein were used as negative controls.

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Chapter 6

GENERAL DISCUSSION

General Discussion Contents

Signaling Pathways As Targets For Cancer Treatment

Despite significant progresses in the treatment of human cancer, novel, more efficient and specific therapies are clearly necessary. Various facts contribute to this assessment: 1) conventional therapies have generally reached their limits and no significant improvements of efficacy and therapeutic index are expected; 2) some cancers respond poorly to induction therapies and no clinical remission is achievable; 3) for several malignancies, the relapse rate is high and, very often, is associated with a bad prognosis, with some cancers remaining largely incurable; 4) conventional therapies are commonly associated with severe complications, which can be particularly damaging for pediatric cancer survivors, and; 5) conventional therapies are often based on empirical facts rather than targeting mechanisms scientifically-proven to be essential for cancer development. It is our contention that the characterization of the signaling pathways that confer tumor cells with survival and proliferative advantages is critical for the development of novel, more specific and efficient strategies for the treatment of cancer.

Indeed, the quest for more effective and highly specific therapies has already placed signaling pathways critical for cell survival, proliferation and/or differentiation under increasing scrutiny. For example, *in vitro* studies have shown that the proliferation of ALL cells can be inhibited by selectively blocking the activation of JAK tyrosine kinases [497]. Small compounds, such as beta-lapachone and taxol, which target cell cycle regulatory molecules, also have been shown to inhibit tumor cell proliferation *in vitro*, and to induce tumor regression *in vivo* [498]. In recent years, intense research has resulted in the development of several other new agents that target altered signal transduction pathways in various types of tumors. The validity of this approach has recently been demonstrated by a clear success story in the treatment of chronic myelogenous leukemia (CML). One of the primary biochemical anomalies caused by the disease-specific fusion oncogene Bcr/Abl in CML is the cytokine-independent activation of the JAK/STAT pathway [499]. In normal cells, this pathway mediates the intracellular response to cytokine stimulation and its constitutive activation by Bcr/Abl plays a crucial role in CML oncogenesis. This knowledge has provided strong support for the generation of strategies

that specifically aim at blocking the activity of Bcr/Abl tyrosine kinase, and led to the development of STI 571, a specific inhibitor of the tyrosine kinase Abl, which abrogates the *in vitro* proliferation of Bcr/Abl-positive leukemic cells and induces clinically significant responses in CML patients [500,501]. The clinical efficacy of STI 571, now an approved treatment for CML [502], and currently undergoing a phase III randomized clinical trial [503], eloquently demonstrates that strategies targeting key signaling molecules via specific inhibitors can be successfully translated to the clinic and used in the actual treatment of cancer patients.

A profound knowledge of the signaling pathways that normally regulate the processes that are affected in a malignant cell might be critically important to overcome another obstacle frequently associated with cancer treatment. It is well established that all forms of cancer therapy have the risk of selecting drug-resistant clones of malignant cells. Therefore, the most effective therapeutic strategies are likely to be combinatorial, affecting several targets specific to the cancer in a coordinate way [105]. Such strategies may not even require each component to be specific for the malignant cells, so long as the combination provides selective inhibition of the tumor. The development of these refined modalities of combined treatment will necessarily require an in-depth characterization of the molecular signaling mechanisms that lead to cancer progression.

Despite the significant success recently achieved in the treatment of T-ALL, this malignancy remains an important therapeutic challenge, since a considerable number of patients are still not cured by conventional therapies, and despite the achievements concerning event-free survival [31], the actual cure rates are considerably lower [504]. Moreover, treatment efficacy is only achieved with intensive chemotherapy, which is often accompanied by toxicities that can lead to severe and long-term complications particularly troublesome in children. Finally, available therapies are significantly less effective in treating adult T-ALL, with survival rates significantly lower than for pediatric ALL [17,31].

T-ALL cells proliferate in discrete microenvironments where they are exposed to exogenous stimuli, such as IL-7, and in which they acquire a selective survival and proliferative advantage. In this work, we analyzed the IL-7-mediated signal transduction pathways and their effect on survival and cell cycle progression of T-ALL cells. The

ultimate goal of the project was to evaluate the possible role of this cytokine in the pathophysiology of T-ALL, and to identify putative molecular targets for the development of specific therapeutic strategies for the treatment of this malignancy.

The majority of our studies were performed using primary T-ALL cells, a rare approach in the field of cancer signaling, since it is considerably more demanding and time-consuming than using continuous cancer cell lines, and is very often limited by the availability of a finite number of malignant cells. However, since the currently available T-ALL cell lines, as most tumor cell lines, have accumulated genotypic alterations and are often functionally quite distinct from the leukemic cells collected from patients at presentation, it is our believe that by overcoming the difficulties of handling primary leukemic T-cells we gained in "physiological insight" regarding the biology of T-ALL. This is particularly important for studies aiming to the identification of biologically essential targets and the development, screening and pre-clinical validation of specific inhibitory agents.

Our studies have demonstrated that T-ALL cells proliferate in response to all cytokines that signal through the common gamma chain, especially IL-7 and IL-4 (Chapter 2), and that downregulation of the cdk inhibitor $p27^{kip1}$ is a key event for not only entry into the cell cycle but also prevention of cell death mediated by IL-7 (Chapter 3). We further observed that IL-7 activates PI3K/Akt, JAK/STAT and ras/MAPK but not p38 MAPK pathway in T-ALL cells (Chapters 2, 4 and 5). Interestingly, IL-7-mediated activation of PI3K/Akt but not MEK/Erk pathway seems to be mandatory for $p27^{kip1}$ downregulation, increased Bcl-2 expression, cell cycle progression and survival of T-ALL cells (Chapter 5). The importance of IL-7 for T-ALL cell expansion was further demonstrated by the establishment and characterization of the IL-7-dependent cell line TAIL7 (Chapter 4).

The Effect Of Cytokines That Signal Through γ_C On T-ALL Cell Proliferation

In Chapter 2, we demonstrated that γ_c-dependent cytokines induce the proliferation of primary T-ALL cells. This study represents the first comprehensive analysis of the effect of this family of cytokines on primary leukemic T-cells (IL-21 was not included, since it was cloned when this work was already in an advanced stage of execution). Previous studies evaluated a very small number of samples and/or focused on solely one or two of these cytokines (see references in Chapter 2). In our work, we used a reasonable number of specimens (n=25) from patients with high leukemia content, and in addition to the previously evaluated IL-2, IL-4 and IL-7, we have also studied IL-9 and IL-15. To our best knowledge, this represents the first study evaluating the impact of IL-9 and IL-15 in T-ALL. Besides the evaluation of the independent effect of each cytokine, we also tested cytokine combinations and demonstrated that synergisms can occur between them. This might be biologically relevant in the microenvironment where the leukemic cells expand.

IL-7 was the cytokine that most frequently and potently induced proliferation of T-ALL primary cells, without inducing their differentiation. A previous study using a similar number of patients [96], reported that IL-7 mediated survival but not proliferation of T-ALL cells. However, this study did not assess DNA synthesis and cellular proliferation. Instead, the authors estimated proliferation of T-ALL cells by evaluating cell cycle distribution at 24 hours of culture. In Chapter 3, we examined not only cell cycle progression (by propidium iodide staining) and cell proliferation (by thymidine incorporation), but most importantly, the molecular events related to cell cycle regulation triggered by IL-7. We observed that cdk activation, hyperphosphorylation of Rb and subsequent cell cycle progression of T-ALL cells in response to IL-7 occurs later in culture. Our results are in agreement with earlier studies that used either thymidine incorporation assessment or blast colony formation to determine IL-7 responsiveness of T-ALL cells (see Chapter 2).

Because IL-7 is the only γ_c-dependent cytokine that has an unambiguous non-redundant role during T-cell development and several lines of evidence point to its

involvement in T-cell leukemogenesis (see Chapter 1 and 2), our subsequent studies focused on IL-7-mediated cellular and molecular events on T-ALL.

$p27^{kip1}$: The Key To IL-7-Promoted Survival And Proliferation Of T-ALL Cells

The cyclin-dependent kinase inhibitor $p27^{kip1}$ was first identified as an inhibitor of the protein kinase activity of G1 cyclin/cdk holoenzymes which could be modulated by external signals [505-507]. The key role of $p27^{kip1}$ in the regulation of G1 cell cycle progression was subsequently demonstrated by numerous studies (reviewed in Ref. [508]) and reflected in the phenotype of $p27^{kip1}$ deficient mice, which display increased body size as a consequence of increased cell number, and occurrence of pituitary tumors [509-511]. Nonetheless, during hematopoiesis, or under particular cellular conditions, $p21^{cip1}$ seems to replace $p27^{kip1}$ as the main regulator of cell cycle progression [512-514]. Moreover, there is evidence that functional collaboration between distinct cdk inhibitors is tissue-specific [515], indicating that cell cycle, as well as tumor suppression, can be differently regulated in different cell types.

We have shown in Chapter 3 that IL-7-cultured T-ALL cells not only upregulated Bcl-2 expression, which was essential for survival, but also progressed in the cell cycle, resulting in upregulation of cyclin D2 and cyclin A, induction of cdk activity and hyperphosphorylation of Rb. Downregulation of $p27^{kip1}$ correlated with these effects and was mandatory for IL-7-mediated cell cycle progression. Forced expression of $p27^{kip1}$ prevented Rb hyperphosphorylation and cell cycle progression. Notably, it also reversed IL-7-stimulated upregulation of Bcl-2 and promotion of viability. The $p27^{kip1}$ gene was not originally believed to function as a tumor suppressor, because both alleles were not found to be deleted or silenced in cancer cells [139]. Presently, there is compelling evidence that $p27^{kip1}$ has a critical role in preventing cancer development and is haplo-insufficient for tumor suppression. Mice with hemizygous or homozygous deletions of $p27^{kip1}$ develop spontaneous tumors and the rate of tumor formation depends on the $p27^{kip1}$ gene copy number (p27 -/- > p27 +/- > p27 +/+) [516,517]. *In vitro* data further implicate $p27^{kip1}$ in

the process of neoplastic transformation, since oncogenes such as Ras and Myc can inhibit $p27^{kip1}$ activity and/or expression [171,518,519]. Although the $p27^{kip1}$ gene is rarely mutated or deleted in tumor cells, reduced protein levels have been observed in multiple human malignancies and strongly correlate with prognosis (e.g., Refs. [520-526]). Interestingly, homozygous inactivation of $p27^{kip1}$, although probably occurring very rarely, has been described in one T-ALL patient [101], and hemizygous deletions of $p27^{kip1}$, resulting in decreased but not absent protein levels, was reported to occur as a consequence of 12p chromosomal deletions in childhood ALL [101]. Moreover, a recent study has demonstrated that $p27^{kip1}$ may have prognostic value in childhood B- and T-ALL. Relapse could be predicted by $p27^{kip1}$ protein levels during the first 48 hours of treatment, and $p27^{kip1}$ expression at 48 hours of corticotherapy was associated with significant differences in the duration of disease-free and overall survival [527]. Our studies show that IL-7 induced proliferation and survival of T-ALL cells by downregulating $p27^{kip1}$ expression, and support the notion that $p27^{kip1}$ is a tumor suppressor gene, not only because it works as a negative regulator of cell cycle progression but also because it is involved in induction of apoptosis by downregulating the levels of Bcl-2 protein.

Opposite results have been reported regarding the effect of $p27^{kip1}$ on cell viability (e.g., Refs. [430,528-530]). This indicates that the ability of $p27^{kip1}$ to induce or prevent apoptosis is probably developmentally regulated and/or cell-type specific and, most importantly, dependent on the cellular context. In this regard, it has been shown that increased $p27^{kip1}$ expression is associated with apoptosis of HL60 human leukemic cells when c-Myc levels remain elevated, but not when exit from the cell cycle and $p27^{kip1}$ upregulation are accompanied by c-Myc protein decline [531]. These results also suggest that in the former case, apoptotic cell death might have resulted from conflicting signaling events. The abnormal simultaneous presence of proliferative (c-Myc) and anti-proliferative ($p27^{kip1}$) activities could trigger the apoptotic program. One cannot exclude that this might also be the case in our experimental conditions, since forced sustained expression of $p27^{kip1}$ by using exogenous VP22/p27 fusion protein can theoretically lead to the expression of $p27^{kip1}$ beyond the restriction point and G1, therefore originating the concomitant presence of cell cycle stimulating and inhibitory signals during the subsequent phases of the cycle. However, we also used Rapamycin to elevate the

endogenous levels of $p27^{kip1}$ and induce an effective G1 arrest, thereby preventing the occurrence of such conflicting signals. Rapamycin is known to induce upregulation of $p27^{kip1}$ and G1 cell cycle arrest without mediating apoptosis [158]. Since VP22/p27 and Rapamycin had very similar effects in downregulating IL-7-promoted viability of T-ALL cells, our observations strongly indicate that $p27^{kip1}$ can induce apoptosis of leukemic T-cells under physiological circumstances.

Several studies have provided direct evidence that high levels of $p27^{kip1}$ can induce apoptosis in normal and cancer cells [161,430,530,532], whereas for example overexpression of $p21^{cip1}$ and $p19^{INK4D}$ cannot [530]. Other studies associate $p27^{kip1}$ upregulation with drug-induced apoptosis of different tumor cell types [533,534], including a T-ALL cell line [535]. However, the apoptotic effect of $p27^{kip1}$ appears to be indirect and the mechanisms by which cell death occurs were not explored. A previous report has shown that Bcl-2 ectopic expression can protect HeLa cells from apoptosis mediated by $p27^{kip1}$ overexpression [532], but did not determine whether Bcl-2 could be affected by $p27^{kip1}$. Interestingly, DP thymocytes that are undergoing thymic selection, which are not proliferating and therefore must have high $p27^{kip1}$ expression, express low levels of Bcl-2 and are more prone to apoptotic cell death [211]. We showed that downregulation of $p27^{kip1}$ is required, at least in part, for IL-7-mediated increase in Bcl-2 protein levels and prevention of apoptosis in T-ALL cells. Our results, although providing an explanation for the pro-apoptotic effects of $p27^{kip1}$, raise an interesting question which merits some speculation: how does $p27^{kip1}$ regulate Bcl-2 protein expression? A possible explanation results from data showing that $p27^{kip1}$ associates with c-Jun and JunD co-activator Jab1. This association promotes nuclear export of the Jab1/$p27^{kip1}$ complex [173,536] and consequently diminishes transcription by AP-1 in T-cells [536]. Although AP-1-mediated transcription has often been implicated in apoptosis, some reports suggest that AP-1 can induce Bcl-2 expression [211,494]. Therefore, high levels of $p27^{kip1}$ could mediate downregulation of Bcl-2 expression and induce apoptosis via impairment of AP-1 transcriptional activity. Another possibility arises from the fact that high $p27^{kip1}$ protein levels are linked to Rb hypophosphorylation and E2F inhibition. IL-7-mediated downregulation of $p27^{kip1}$ results in cyclin/cdk activation. Activated cyclin/cdk complexes phosphorylate Rb, which becomes inactive thereby releasing E2F

transcription factors from Rb inhibitory constraints. E2F transcriptional activity could then result not only in expression of S-phase genes but also of Bcl-2. In support of this hypothesis, it was recently found that transfer of E2F family members to human glioma cells results in transcriptional up-regulation of Bcl-2 [537]. Future studies will reveal whether the same mechanism occurs in T-ALL cells.

There is evidence that IL-7 controls other regulators of apoptosis such as Bax and Bcl-X_L. Bax-deficient mice have lymphoid hyperplasia with total number of thymocytes being almost two-fold higher than normal controls [538], and withdrawal of IL-7 induces Bax translocation from cytosol to mitochondria in mouse thymocytes [247]. IL-7 upregulates Bcl-X_L in activated primary human T-cells, rescuing them from apopotosis induced by IL-2 withdrawal [539], although Bcl-X_L might not be relevant for thymocyte development, since its pattern of expression is opposite to Bcl-2 and IL-7R during T-cell ontogeny. Thus, the fact that culture with IL-7 does not induce any significant changes in expression of Bcl-2 family members, other than Bcl-2 itself, does not necessarily implicate that IL-7 could not regulate their activity in T-ALL by affecting their cellular localization, phosphorylation and/or activation status. That could be the case of Bad, which was shown to be phosphorylated and inactivated by PI3K/Akt pathway [119], which in turn is triggered by IL-7. However, in preliminary studies using a phospho-specific anti-Bad antibody we were not able to detect any IL-7-induced Bad phosphorylation (data not shown). The possibility of subcellular redistribution of Bcl-2 family members, with particular emphasis to mitochondrial relocalization, without alteration of protein levels has been reported in T-ALL and associated with modulation of apoptosis in response to TNF-α [540]. Moreover, we cannot exclude the possibility that other members of Bcl-2 family not studied here (Bim, A1, etc) take part in IL-7-mediated viability signals. However, the mandatory character of Bcl-2 expression for IL-7-induced viability strongly indicates that Bcl-2 is the most critical effector of IL-7 regarding protection from apoptosis in T-ALL cells.

Our results regarding the role of $p27^{kip1}$ in IL-7-mediated proliferation and viability are schematically summarized in Figure 11.

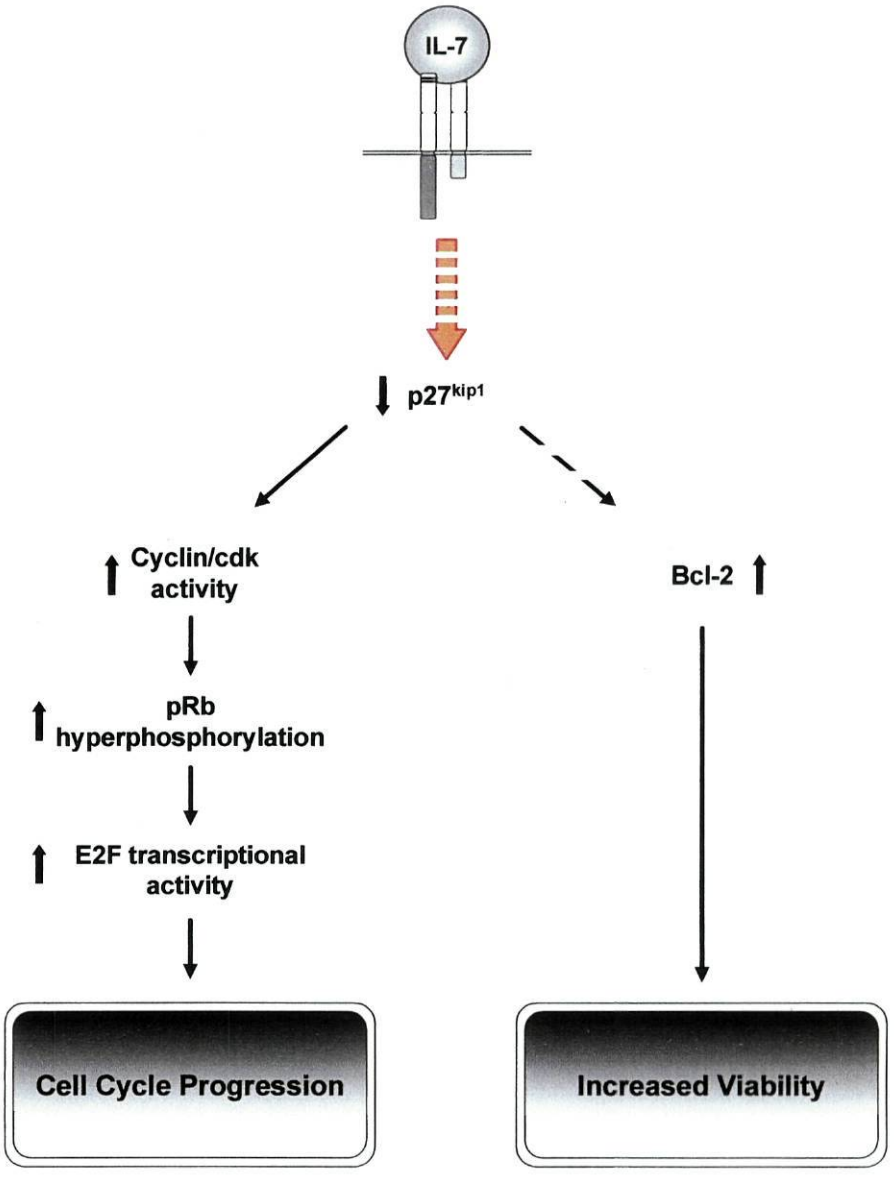

Figure 11. IL-7-mediated cell cycle progression and viability in T-ALL. Stimulation of T-ALL cells with IL-7 induces $p27^{kip1}$ downregulation, which in turn is mandatory for cyclin/cdk enzymatic activity, Rb phosphorylation and consequently E2F transcriptional activity and cell cycle progression. IL-7-promoted decrease in $p27^{kip1}$ protein expression is responsible, at least in part, for upregulation of Bcl-2 and viability of T-ALL cells.

PI3K/Akt Pathway: The Bridge Between IL-7 And $p27^{kip1}$ In T-ALL

Once we established the cell cycle mechanisms via which IL-7 induces expansion of T-ALL cells, we sought to determine the cellular signaling events that link the cell surface triggering of IL-7R by IL-7 to the downstream regulation of cell cycle progression and viability. In other words, which pathway(s) activated after IL-7 stimulation could mediate downregulation of $p27^{kip1}$ and upregulation of Bcl-2 ?

As shown in Chapter 5, our results using pharmacological inhibitors indicate that IL-7-mediated activation, proliferation and survival of T-ALL cells are dependent on PI3K/Akt activation. Accordingly, downregulation of $p27^{kip1}$ and increased expression of Bcl-2 depend upon activation of PI3K/Akt. In fact, the first hint that PI3K/Akt pathway could be the critical mediator of IL-7 molecular and functional effects on T-ALL resulted from our observation that Rapamycin blocked proliferation and diminished survival promoted by IL-7 (Chapter 3). Rapamycin upregulates $p27^{kip1}$ and exerts its inhibitory effects by binding to the immunophilin intracellular receptor FKB12 and, as a complex with FKB12, inhibiting the activities of mTOR (reviewed in [541]). Akt/PKB has been reported to directly phosphorylate mTOR (see Chapter 1 for references). Thus, Rapamycin appears to prevent IL-7-mediated events in T-ALL by inhibiting a PI3K/Akt/mTOR pathway that is involved in downregulation of $p27^{kip1}$. Although the mechanisms by which mTOR induces decreased $p27^{kip1}$ protein expression are presently unknown, it has been suggested that mTOR could contribute to increased translation rates of mRNA species (e.g. Skp2) whose protein product is involved in the degradation of $p27^{kip1}$ by the ubiquitin-proteasome system [541]. However, the end-result of Rapamycin action, $p27^{kip1}$ upregulation, could possibly also be achieved by targeting other Akt/PKB-associated mechanisms. Indeed, the roads that lead from Akt/PKB activation to downmodulation of $p27^{kip1}$ are multiple and involve several downstream targets besides mTOR (Figure 12). We showed that PI3K-dependent activation of Akt by IL-7 leads to GSK-3α/β *in vitro* phosphorylation, and provided evidence that the same effect is probably exerted by Akt *in vivo*. GSK-3 phosphorylation results in its inhibition and could lead to c-Myc protein stabilization and consequent $p27^{kip1}$ downregulation [542,518]. Another possible mechanism involves inhibition of Forkhead family members (FKHR,

FKHRL1 and AFX). All of them have been implicated in transcription of $p27^{kip1}$, and Akt/PKB inhibits their activity by phosphorylating them at critical residues. We showed that FKHR is constitutively phosphorylated/inhibited in TAIL7 cells and does not appear to be involved in IL-7-mediated effects. Nonetheless, we did not evaluate whether the other Forkheads, FKHRL1 and AFX, could be functional downstream targets of IL-7-activated Akt/PKB in T-ALL. Finally, a new and very direct role for Akt/PKB in $p27^{kip1}$ regulation has been recently established by different groups [170,543-545]. Akt/PKB directly phosphorylates $p27^{kip1}$ at several residues, thereby retaining $p27^{kip1}$ in the cytoplasm (threonine 157), promoting its nuclear export (serine 10 and threonine 198), and/or leading to $p27^{kip1}$ protein degradation (threonine 187).

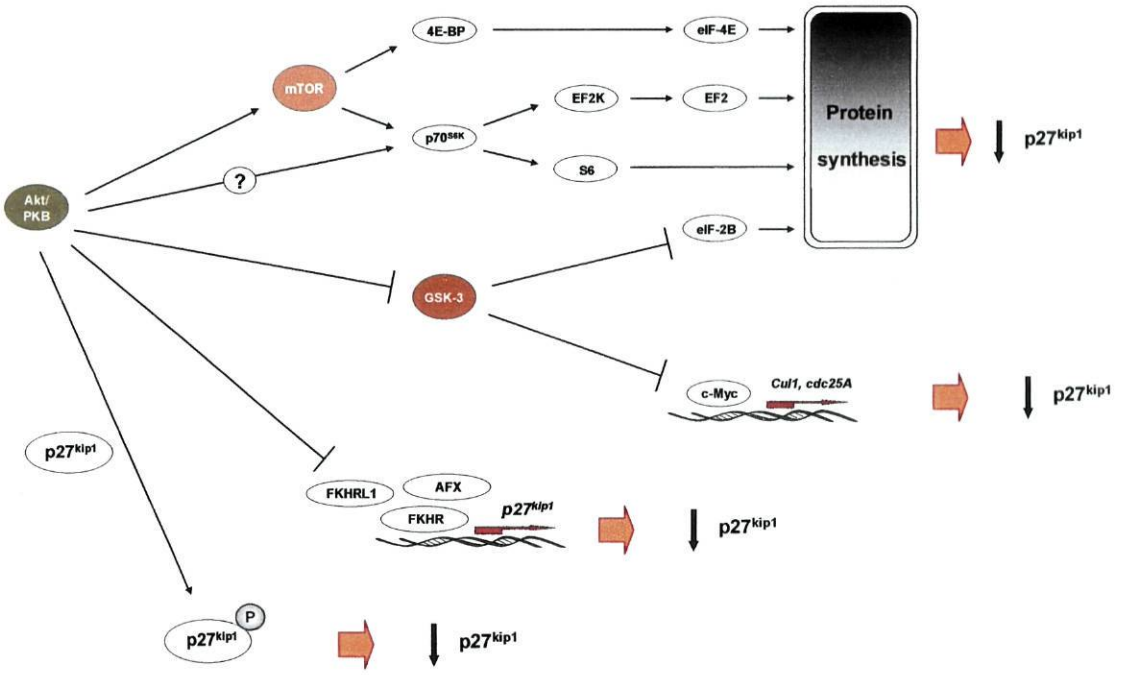

Figure 12. Akt/PKB mediates $p27^{kip1}$ downregulation by multiple mechanisms. Akt/PKB directly phosphorylates mTOR and possibly p70S6K, resulting in activation of translation initiation factors and increased protein translation. Although the exact mechanisms are not known, this ultimately leads to a decrease in $p27^{kip1}$ protein levels. Akt/PKB-mediated inhibition of GSK-3 contributes both to increased protein translation and c-Myc activity. c-Myc induces $p27^{kip1}$ sequestration and degradation. Members of the Forkhead family (FKHR, FKHRL1, AFX) transcriptionally induce $p27^{kip1}$. Akt/PKB inhibits the activity of Forkheads. Recently, it has been shown that Akt/PKB directly phosphorylates $p27^{kip1}$, thus promoting its nuclear export and protein degradation by the ubiquitin/proteasome system.

PI3K/Akt Pathway Aberrations In T-ALL ?

In view of the growing awareness of the importance of PI3K/Akt pathway in tumorigenesis [546], and the knowledge that several leukemic T-cell lines show constitutive activation of PI3K and Akt as a result of PTEN defects [481], it is rather surprising that no studies have been published evaluating the activation status and biological significance of this pathway in primary T-ALL cells. TAIL7 cell line does not have constitutive activation of Akt/PKB (Chapter 5). However, we unexpectedly observed that in unstimulated TAIL7 cells FKHR is constitutively phosphorylated at a residue (serine 256), which is a normal target for Akt/PKB-mediated phosphorylation. Yet, FKHR phosphorylation was not significantly altered by either IL-7 stimulation or the addition of LY294002, indicating that FKHR phosphorylation is independent of PI3K/Akt pathway. A tempting hypothesis is that FKHR constitutive phosphorylation could be due to deregulation of PP1A and/or PP2A activity. As mentioned in Chapter 1, these proteins dephosphorylate the Akt target Bad, hence raising the question of whether they can also dephosphorylate FKHR. If that is indeed the case, then inactivation of PP1A/PP2A by deletion or mutation could result in FKHR constitutive phosphorylation without the involvement of PI3K/Akt. Since PP1A and PP2A were suggested to act as tumor suppressors and deregulation of their activity has been associated with tumorigenesis, it would be interesting to evaluate whether these phosphatases play a significant and yet unexplored role in T-cell malignancies.

IL-7-Mediated Mechanisms Of Proliferation And Viability In T-ALL Cells

Based on our experimental results and available data concerning IL-7 signaling, we suggest the following model for IL-7-mediated regulation of cell cycle progression and viability in T-ALL (Figure 13). Upon IL-7 stimulation, Jak3 is activated (Chapters 2 and 4, and Ref. [318]) and phosphorylates STAT1, STAT3 and STAT5 (Chapters 4 and 5) leading to their activation. MEK/Erk pathway is also activated in response to IL-7 by

unknown mechanisms (Chapters 4 and 5). Jak3 also phosphorylates and consequently activates PI3K [294]. This appears to be the most critical event mediated by Jak3 in IL-7-stimulated T-ALL cells since inhibition of PI3K by the specific inhibitor LY294002 is sufficient to completely abrogate IL-7-mediated proliferation and viability (Chapter 5). In contrast, inhibition of MEK/Erk pathway did not affect IL-7-mediated effects. Although we did not evaluate the functional outcome of STAT activation, it is predictable that Jak/STAT pathway plays no major role in IL-7-mediated survival and proliferation of T-ALL cells, as it did not compensate for PI3K/Akt inhibition. Thus, the critical role of Jak3 in IL-7-promoted survival and/or proliferation of primary T-ALL cells (Chapter 2) and TAIL7 cell line (Chapter 4) is most probably mediated via activation of PI3K, and not through STATs. PI3K induces Akt/PKB activation and subsequently Akt/PKB inhibits GSK-3 by direct phosphorylation (Chapter 5). These events are associated with $p27^{kip1}$ downregulation, pRb hyperphosphorylation and cell cycle progression (Chapters 3 and 5). Decreased levels of $p27^{kip1}$ are necessary for upregulation of Bcl-2 protein expression, which in turn is critical for IL-7-mediated viability response in T-ALL (Chapter 3). The complementary outcome of $p27^{kip1}$ downmodulation on both cell cycle progression and viability originates a prolonged proliferative effect (Chapter 2) that allows for T-ALL cell expansion.

Is IL-7 Biologically Important For The *In vivo* Expansion Of T-ALL?

We analyzed how IL-7 modulates the behavior of T-ALL *in vitro*, and have shown that IL-7 can increase viability and induce proliferation of the leukemic cells. However, the question remains of whether IL-7 has an actual regulatory role *in vivo* and significantly contributes to the pathophysiology of this malignancy. While no answer to this question can be achieved until the appropriate experiments are conducted, we argue that indeed IL-7 can be biologically significant for the progression of T-ALL. In the following paragraphs, and using as foundation our own observations, we will present the rationale and the circumstantial evidences that support our hypothesis.

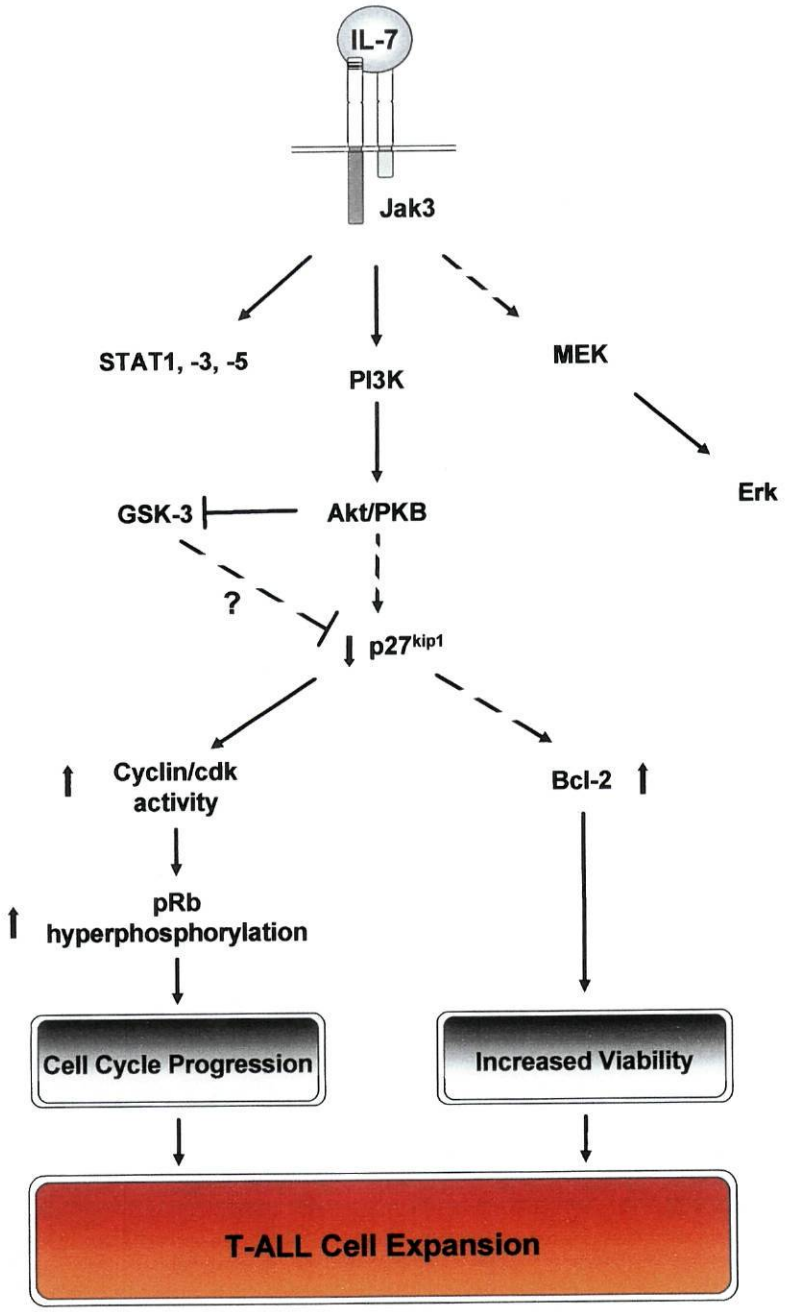

Figure 13. A model for IL-7-mediated mechanisms of T-ALL cell expansion. Binding of IL-7 to the IL-7R induces activation of Jak3, STATs, MEK/Erk and PI3K/Akt. The role of MEK/Erk pathway and STATs in IL-7-mediated signaling in T-ALL is currently under investigation, but neither of these pathways appears to be essential for cell cycle progression or viability. IL-7 mediates Akt/PKB activation in a PI3K-dependent manner. Subsequently, Akt/PKB inhibits GSK-3 by direct phosphorylation. These events lead to $p27^{kip1}$ downregulation, pRb hyperphosphorylation and cell cycle progression. Decreased levels of $p27^{kip1}$ also mediate upregulation of Bcl-2 protein expression, which is critical for IL-7-induced viability in T-ALL. Cell cycle progression and viability support a prolonged proliferative effect that allows for T-ALL cell expansion.

Despite the aggressiveness of the malignancy, T-ALL cells are not isolated entities that survive and proliferate indiscriminately without any interference from the surrounding elements. If that was the case, ex-vivo cultured primary T-ALL cells would expand inexorably. However, it is common knowledge to leukemia researchers that T-ALL cells almost inevitably enter spontaneous apoptosis *ex vivo*, even when cultured in rich culture conditions. Moreover, the fact that the rate of spontaneous apoptosis of primary T-ALL cells can be reduced by cytokines or stromal cells [96,547], has lead led to the suggestion that microenvironmental signals are continuously rescuing the leukemic cells *in vivo* [548]. Thus, the malignant T-cells probably expand *in vivo* as a result of a combination of cell-autonomous and microenvironmental events that collaborate to confer a selective advantage to the malignant cells in detriment of their normal counterparts. Stroma-produced survival factors are likely to be fundamental for the pathophysiology of T-ALL.

As mentioned at several instances in previous chapters, IL-7 plays a critical role as a survival factor during early T-cell differentiation and it is produced in the tissues where T-cell precursors develop, i.e. in the bone marrow and subsequently the thymus. A stimulatory effect of IL-7 on proliferation of normal T-cell precursors has also been reported by several studies, whereas the ability of IL-7 to actively induce differentiation has never been convincingly demonstrated, except for γδ T-cells. How can a factor that is fundamental for normal development be fundamental for expansion of abnormal cells? Apparently, for the exact same reasons. In other words, IL-7 is produced in the microenvironments where the leukemic clone develops, and IL-7 is a survival and growth factor for the leukemic cells, without being able to overcome their differentiation block. In addition, IL-7 can act as an oncogene *in vivo*, since an IL-7 transgene promotes malignant transformation of lymphocytes resulting in the development of B and T-cell lymphomas. This also implicates that IL-7 can support the expansion of malignant lymphocytes *in vivo*, a property that can certainly affect T-ALL cells.

Another question then arises: how does a cytokine that similarly stimulates malignant T-cells and their normal counterparts benefit the former more than the latter? The answer lies most probably within the cells. As we mentioned in Chapter 1, a clone carrying an oncogenic mutation might respond differently than its normal counterpart to

the same stimuli. For example, a clone with a mutation leading to augmented proliferation will have a considerable advantage when receiving external signals mediating survival. Likewise, mitogenic signals will contribute to the expansion of a malignant population possessing a mutation that solely augments the viability but not the proliferation of the transformed cells. An actual portray of this occurs in human follicular B-cell lymphoma, where a translocation leads to upregulation of Bcl-2 expression. This results in an indolent tumor whose expansion is initially dependent upon exogenous signals. Subsequently, the malignant cells undergo additional mutations and a substantially more aggressive tumor emerges [105]. This example also demonstrates that external signals, such as IL-7, can offer time to the leukemic cells for the acquisition of additional advantageous mutations. A similar phenomenon to the one described above can theoretically occur in T-ALL. For example, leukemic cells with a t(8;14)(q24;11) translocation would present c-Myc overexpression and as a consequence would tend to have an increased rate of proliferation, as compared to normal developing T-cell cells. However, it is known that many mechanisms that drive cell proliferation trigger or sensitize a cell to apoptosis [549], and that is the case for c-Myc-induced proliferation, which also leads to apoptosis [550]. Since the leukemic clone arises in an IL-7-rich environment, the easily-available external survival signal provided by IL-7 would prevent c-Myc-mediated apoptosis and allow the overgrowth of the leukemic clone at the expense of normal thymocytes. In fact, it has been demonstrated that some external factors are able to inhibit apoptosis induced by c-Myc overexpression [550,551]. IL-7 might be utilized by T-ALL cells to promote survival and/or proliferation in a way that ultimately leads to clonal expansion and progression of the disease.

In the future, analysis of IL-7 and c-Myc (or other critical T-ALL oncogene) double transgenic mice, or administration of IL-7 to c-Myc transgenic mice could prove our hypothesis that IL-7 confers a selective advantage to leukemic cells *in vivo*. Alternatively, culture experiments using overexpression of c-Myc or another critical T-ALL oncogene in TAIL7 cells resulting in abrogation of IL-7-dependency would support the view that IL-7 collaborates with oncogenes for leukemogenesis. In accordance with this notion, it has been suggested that IL-7 could be one of several synergistic events leading to malignant conversion of pre-B-cells [552]. Notably, co-infection of IL-7 and v-

Ha-ras oncogene into murine bone marrow generated a pre-B lymphoid outgrowth that was tumorigenic. Although overexpression of IL-7 and v-Ha-ras were not individually sufficient to induce malignant transformation, their co-expression was synergistic in yielding highly neoplastic pre-B-cell lines [553].

A recent study has shown that normal and tumor cells differ in their requirement for $p27^{kip1}$ in TGF-β mediated cell cycle arrest [554]. Using antisense oligonucleotides to inhibit $p27^{kip1}$ expression in finite lifespan human mammary epithelial cells (HMECs) and in cancer-derived lines, the authors demonstrate that HMECs, but not the tumor cell lines, maintain G1 arrest after $p27^{kip1}$ downregulation through a compensatory accumulation of $p21^{cip1}$ and p130 in cyclin E/cdk complexes. Their data indicate that malignant transformation may alter the role of $p27^{kip1}$ from a redundant to an essential inhibitor of cell cycle progression. In turn, this implicates that external proliferative factors, such as IL-7, may even be more efficient in inducing cell cycle progression in malignant cells than in their normal equivalents, as exogenous stimulation may overcome the last obstacle - $p27^{kip1}$ - to tumor progression, whereas in the normal cells other inhibitors can compensate for $p27^{kip1}$ under some circumstances.

An unexplored and particularly exciting putative link between IL-7 and T-ALL transformation concerns the finding that the T-ALL oncogenic transcription factor TAL1/SCL (see Chapter 1) has a consensus phosphorylation site for Akt/PKB [555]. This has two possible implications: 1) Akt/PKB might phosphorylate TAL1/SCL and thereby modulate its activity, and 2) activation of PI3K/Akt pathway either by IL-7 stimulation or as a consequence of genetic alterations might lead to modulation of TAL1/SCL activity. At this point, it is important to recall that phosphorylation mediated by Akt/PKB can induce transcription factor activation (e.g., CREB). Taking this into consideration, and the fact that both TAL1/SCL and Akt/PKB are tumorigenic factors, it is probable that the hypothetical Akt/PKB-mediated phosphorylation of the T-ALL transcription factor would result in its activation. Additionally, it has been suggested that hyperphosphorylation of inappropriately expressed TAL1 is highly oncogenic [57]. Future studies are required to evaluate whether an IL-7/PI3K/Akt/TAL1 pathway does exist and confirm the ability of IL-7 to regulate T-ALL tumor progression via modulation of the activity of leukemogenic transcription factors.

Interestingly, IL-7 might contribute to leukemogenesis also via activation of MEK/Erk pathway. Although activation of this pathway is not directly required for proliferation or viability of T-ALL cells (Chapter 5), there is evidence of its involvement in regulation of different transcription factors associated with T-cell leukemogenesis. TAL1 and TAL2 are phosphorylated at specific serine residues by Erk1, thereby modifying their DNA binding activities, and during erythroid differentiation, erythropoietin, which activates MEK/Erk and PI3K pathways, induces phosphorylation of TAL1 (see Chapter 1 for references). It has also been shown that TCR-mediated signals in normal thymocytes downregulate the DNA-binding capacity of E2A transcription factor, via ras/MEK/Erk pathway [556]. E2A opposes the effects of TAL1 and LYL1, and E2A deficiency leads to abnormalities in T-cell development and rapid development of T-cell malignancies [557,558]. Activation of MEK/Erk pathway by IL-7 in T-ALL might, therefore, have the subtle but significant effect of augmenting the phosphorylation and activation of oncogenic transcription factors and/or diminishing the activity of tumor suppressor transcription factors, thereby promoting the T-cell leukemic phenotype.

Finally, IL-7 can be implicated in leukemogenesis at another biological level. As mentioned in Chapter 1, IL-7 *in vitro* stimulation of human thymocyte cell lines has been shown to consistently upregulate VEGF gene expression [261], suggesting that IL-7-stimulation of malignant thymocytes could indirectly contribute to the production of angiogenic factors and result in *de novo* angiogenesis.

Altogether, the data presented above provide strong evidence, albeit mostly circumstancial, supporting our contention that IL-7 can act as an oncogenic growth factor in T-ALL.

Therapeutic Implications Of The Present Work

The clinical value of agents targeting specific molecular abnormalities associated with signal transduction has been convincingly established in cancer treatment with the success of Herceptin® (trastuzumab), a monoclonal antibody against the receptor

tyrosine kinase Her-2/neu, and especially STI 571 (Gleevec^{TM}), a small molecule inhibitor of Bcr-Abl [559]. Years of research on the cellular and molecular biology of cancer translated into the development of rationally designed, specifically-targeted therapies, and the enthusiasm created by the clinical evidence of their efficacy is well demonstrated, for example, by the exponentially growing number of papers studying or reviewing the effects of STI 571. Specific targeting of signal transduction molecules is currently a pharmacological reality, and the growing awareness of its potential for the treatment of neoplastic diseases resulted in an increasing number of these agents being under evaluation in clinical trials [559-563]. In contrast to earlier chemotherapeutic tools, which were found and utilized in a rather empirical way, these new agents were developed with support of concrete knowledge on the molecular mechanisms associated with transformation and expansion of a particular type of tumor.

Our work has allowed the identification of several potential targets for the development of novel therapeutic approaches specifically targeting those molecules that were shown to contribute to T-ALL cell expansion by preventing apoptosis and/or stimulating proliferation. Therapeutic strategies aimed to restore, mimic or potentiate $p27^{kip1}$ activity might be extremely useful in T-ALL therapy by preventing cell cycle and inducing apoptosis. Since cyclin E/cdk2 activity targets $p27^{kip1}$ for degradation, the use of cdk inhibitors could be an indirect way of increasing $p27^{kip1}$ protein levels [564,565]. As we have showed, Rapamycin, which leads to the upregulation of $p27^{kip1}$, is capable of blocking cell cycle progression and inducing cell death of T-ALL cells *in vitro*. Furthermore, because Rapamycin inhibits PI3K/Akt/mTOR pathway, it could be of particular usefulness in those possible cases where PI3K/Akt pathway would be constitutively activated. Studies with the Rapamycin derivative CCI-779 in mice have demonstrated the usefulness of this inhibitor in the preventing the growth of PTEN-deficient tumors [396,449], and on-going Phase I and II clinical trials in diverse types of cancer indicate that CCI-779 is an extremely promising tool in cancer therapy, as it is well tolerated at doses that are very efficacious against a variety of tumors [566,567]. Hence, Rapamycin and its derivatives, are also potentially valuable for T-ALL treatment. The use of proteasome inhibitors is a novel and promising approach to cancer therapy, and several Phase II clinical trials are underway in different hematological malignancies [568].

Inhibition of proteasome function results in blockade of cell division in normal cells. However, transformed cells are even more sensitive to the targeting of proteasome function and several studies have demonstrated that proteasome inhibition can induce, or sensitize malignant cells to, apoptosis [569,570]. Although proteasome function affects many other molecules, this provides an interesting link to $p27^{kip1}$ protein expression. In fact, it has been shown that proteasome inhibition induces $p27^{kip1}$ accumulation and apoptosis in transformed, but not in normal, human fibroblasts [571]. Alternatively, a critical signaling pathway mediating $p27^{kip1}$ downregulation in T-ALL, as the IL-7-activated PI3K/Akt pathway, may also constitute a target for therapeutic intervention aiming to reestablish or upregulate the levels of expression of $p27^{kip1}$. In pre-clinical studies, LY294002 has been shown to inhibit tumor growth both *in vitro* and *in vivo* [572], increase the efficacy of ionizing radiation [573], and overcome resistance to some chemotherapeutic agents and enhance the effect of others when used in combinatory regimens [574-576].

We have shown the importance of Bcl-2 for IL-7-mediated survival of T-ALL cells. As a consequence of IL-7 physiological stimulation, it is predictable that Bcl-2 protein levels are elevated even in the absence of particular genetic alterations leading to the same outcome. Thus, inhibition of Bcl-2 anti-apoptotic function might also be a way to differentially affect leukemic cells without significantly affecting their normal counterparts. The use of Bcl-2 antisense oligonucleotides has been used to induce tumor cell apoptosis and sensitize malignant cells to chemotherapy in different cancer types (e.g., Refs. [577,578]) and Bcl-2 antisense therapy is currently being evaluated in ongoing clinical trials [579]. Additionally, cell-permeable peptide inhibitors and small-molecule inhibitors of anti-apoptotic Bcl-2 family members, including Bcl-2, are currently being developed and tested *in vitro* and *in vivo* for their efficacy in preventing tumor cell growth [532,580-583].

Obviously, IL-7 and IL-7R are further targets for the development of therapeutic strategies in T-ALL and other hematological malignancies that show IL-7R expression and responsiveness to IL-7 *in vitro*. This rationale has led to the construction of a fusion protein, DAB389 IL-7, composed of the catalytic and transmembrane domains of diphteria toxin, fused to IL-7. DAB389 IL-7 was shown to be selectively cytotoxic strictly for those cells bearing the IL-7 receptor and its use suggested as a potential

therapeutic tool in the treatment of acute leukemias [584,585]. The feasibility of a similar approach has been demonstrated in clinical trials using intravenous administration of DAB486-IL-2 to patients with refractory haematological malignancies [584]. Blockade of the interaction between IL-7 and its receptor could be achieved through the administration of antibodies against IL-7 and/or IL-7R. This kind of strategy has already been tested with success for insulin, IGF-1 and their receptors in ALL cell lines [586]. In addition, the Her2/Neu-specific antibody Herceptin has been evaluated in phase III clinical trials, showing to significantly enhance the efficacy of chemotherapy when used in combination [587], and is presently an approved treatment for breast cancer [559].

We expect that TAIL7 cell line (Chapter 4) will become a valuable tool not only for dissecting cytokine-mediated signaling pathways in T-ALL but also for testing the efficacy of some of the above mentioned, as well as novel, target-specific inhibitors. The anti-tumorigenic efficacy of such agents will be assessed both *in vitro* and using the NOD/SCID model of human T-ALL. TAIL7 cell line will provide the cell numbers necessary for systematic and reproducible testing, while maintaining most of the characteristics of primary T-ALL cells that other factor-independent highly manipulated cell lines have lost, therefore allowing analyses that should more accurately reflect the biological responses of primary T-ALL cells.

Theodor Boveri predicted almost 100 years ago that cancer was caused by genetic/chromosomal aberrations. Since then, extensive research dedicated to the study of this extremely heterogenous group of diseases has confirmed that prediction. However, knowledge concerning the gene defects associated with cancer does not necessarily contain all the answers that will open the doors to the effective cure of all types of tumors. The levels of complexity are far greater, and are only beginning to be uncovered. Differences in resistance and susceptibility to chemotherapy in patients with the same apparent genetic lesions are a reality that underlines the need to better understand the broader mechanisms involved in tumor development, from the molecule to the cell, and from the cell to the microenvironment that surrounds it. As mentioned early on, tumor progression results from a concatenation of alterations that affect the chromosomes, gene

expression, signal transduction pathways, tumor microenvironment, and the capacity of the immune system to recognize and respond to the neoplastic cells. Signal transduction pathways are particularly fertile in providing useful information to comprehend and hence treat cancer, because they provide the link between the external environment and the cell, and at the same time — probably as a consequence of their critical importance — they represent major targets of the genetic aberrations that constitute the first trigger of oncogenesis.

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