"Learn from yesterday, live for today, hope for tomorrow.
The important thing is to not stop questioning."

Albert Einstein

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ABBREVIATIONS INDEX

αβ T cell: Alpha beta T cell
β-ME: β-mercaptoethanol
ΔΨ**M:** Mitochondrial Transmembrane Potential
γC: Gamma-common chain of the IL-2 receptor family
γδ T cell: Gama delta T cell
3H-TdR: Tritiated Thymidine
7AAD: 7-Aminoactinomycin D
AEBSF: 4-(2-Aminoethyl) Enzenesulfonyl Fluoride Hydrochloride
Akt/PKB: v-akt Murine Thymoma Viral Oncogene Homolog 1 / Protein Kinase B
AnnV: Annexin V
Apaf-1: Apoptotic protease activating factor 1
APC: Allophycocyani
APS: Ammonium Persulfate
BAD: BCL2-associated agonist of cell death
Bcl-2: B-cell CLL/Lymphoma 2
Bcl-XL: Bcl-2 like 1
Bim: BCL2-like 11
BM: Bone marrow
CD: Cluster of differentiation
CDK: Cyclin Dependent Kinases
CDKN1A: Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
cDNA: coding Deoxyribonucleic Acid
CIB1: Calcium and integrin-binding protein 1
CLP: Common lymphoid progenitors
CMJ: Corticomedullary junction
CMP: Common myeloid progenitors
Cyt c: Cytochrome c
DC: Dendritic cell
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic Acid
DN: Double negative
dATP: Deoxyadenosine triphosphate
dNTP: Deoxyribonucleotide
DP: Double positive
EDG: G coupled receptor
EGF: Epidermal growth factor
ERK: Extracellular-signal-regulated kinases
ETP: Early thymic progenitor
FACS: Fluorescence Activated Cell Sorting
FBS: Fetal Bovine Serum
FTY720: Fingolimod
FOXO1/3a: Forkhead family of transcription factors
GABP: GA-binding protein
Gfi-1: Growth factor-indenpendent-1
Glut-1: Glucose transporter 1
GM: Granulocyte/ monocyte
CPCR: G protein-coupled receptors
GSK-3: Glycogen synthase kinase 3
IL-2: Interleukin 2
IL-7: Interleukin 7
IL-7R: IL-7 Receptor
Jak: Janus Kinase

HSC: Hematopoietic Stem Cells
MØ : Macrophages
Mcl-1: Myeloid cell leukemia sequence 1
MFI: Mean Fluorescence Intensity
MHC: Major histocompatibility complex
mRNA: messenger Ribonucleic Acid
Myc: Myelocytomatosis Viral Oncogene Homolog
MkE: Megakaryocyte/ erythrocyte
MPP: Multi potent progenitors
NF-kB: Factor Nuclear kappa B
NGF: Nerve growth factor
NK: Natural killer cells
NLS: Nuclear localization signals
NES: Nuclear export signals
N.d.: No data
PARP: Poly (ADP-ribose) Polymerase-1
PCR: Polymerase Chain Reaction
PH domain: Pleckstrin Homology domain
PI: Propidium iodide
PI3K: Phosphoinositide 3-kinase
PLC: Phospholipase C
PP2A: Protein phosphatase 2A
PTEN: Phosphatase and Tensin Homolog
RT-PCR: Real time polymerase chain reaction
siRNA: Small interfering RNA
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SP: Single positive
S1P: Sphingosine-1-phosphate
S1PR: Sphingosine-1 phosphate receptor
SPHK: Sphingosine kinase
SPL: Sphingosine-1-phosphate lyase
SPP: Sphingosine-1-phosphate phosphatase
SOCS: Suppressor of cytokine signaling
STAT: Signal Transducers and Activators of Transcription
SCZ: Subcapsular zone
T-ALL: T-cell Acute Lymphoblastic Leukemia
TBS-T: Tris-Buffered Saline and Tween 20
TCR: T-cell receptor
TEMED: Tetramethylethylenediamine
T-LGL: T-cell large granular lymphocytic leukaemia
TLSP: Thymic stromal lymphopoietin
TSP: Thymic seeding progenitors
TEC: Thymic epithelial cell
TMRE: Tetramethylrhodamine Ethyl Ester
TRAF2: Tumour necrosis factor receptor-associated factor 2
VEGF: Vascular endothelial growth factor
WBC: White blood cell counts

ABSTRACT

Acute lymphoblastic leukemia (ALL) is the most common malignancy in pediatric patients and is characterized by bone marrow and peripheral blood invasion from malignant lymphoblasts. Approximately 15% of children and 25% of adult ALL cases are of T-cell phenotype (T-ALL), which is associated with high risk and poorer prognosis.

Interleukin-7 (IL-7) and its receptor (IL-7R) are essential for normal T-cell development and homeostasis. However, IL-7/IL-7R-mediated signaling may also partake in leukemia development, as demonstrated by the identification of IL-7Rα gain-of-function mutations in around 9% of T-ALL patients.

Sphingosine Kinase (SPHK) is a lipid kinase that promotes cell viability by phosphorylating sphingosine and thereby regulating the ceramide/sphingosine 1-phosphate (S1P) rheostat. Cancer cells frequently display high levels of SPHK, and SPHK expression has been correlated with cancer patients' outcome. Previous studies have shown that increased SPHK levels are correlated with increased cell viability and inhibition of apoptosis in chronic myeloid leukemia and acute myeloid leukemia.

Here, we show that SPHK is an important player in IL-7-mediated signaling in T-ALL. Initially, we demonstrated that SPHK1 expression was increased in T-ALL cells compared to its normal counterparts. We then hypothesized that SPHK1 could be involved in IL-7-mediated positive effects in T-ALL cells (both IL-7-dependent and IL-7Rα mutant), as well as in normal T-cells. We demonstrated that IL-7 activates SPHK activity without significantly impacting on its expression. SPHK inhibition completely prevented IL-7-mediated activation of PI3K/AKT and STAT5 pathways, suggesting that SPHK activity is fundamental for the activation of IL-7-dependent survival pathways. In accordance, inhibition of SPHK decreased IL-7-dependent maintenance of mitochondrial membrane potential and cell viability. In addition, SPHK was necessary for IL-7-dependent cell cycle progression, with its inhibition inducing an arrest in G0/G1. Finally, SPHK inhibition downregulated CD71 surface expression and cell size in T-ALL.

In summary, our study identifies SPHK1 as an essential modulator of IL-7-mediated signaling in T-ALL, and opens new possible therapeutic approaches by using SPHK pharmacological inhibitors in the treatment of T-ALL patients.

Keywords: T-Cell Acute Lymphoblastic Leukemia, Interleukine-7, Sphingosine Kinase

INTRODUCTION

1.1. An overview of T-cell development

The thymus is organized into four different regions that include the subcapsular zone (SCZ), the cortex, the medulla, and the corticomedullary junction (CMJ, Figure 1). The subcapsular zone is composed by cortical thymic epithelial cells (cTECs), and the cortex has a mixture of cTECs, fibroblasts, and macrophages (MØ). The medulla contains a stromal network of dendritic cells (DCs) and medullary thymic epithelial cells (mTECs). The corticomedullary junction contains a dense network of endothelial cells, which helps the access into the thymus (1). The production of different chemokines, cytokines and ligands is required to interact with T-cell progenitors in development. One of the important cytokines is IL-7, which is produced by stromal cells in thoracic thymus, cervical thymus and bone marrow being required for T cell development and homeostasis (2).

T-cell development occurs in the thymus. Detailed below is a summary of T-cell development in the mouse, where the process is better characterized. The most fundamental steps described here appear to be similar in humans. Thymic seeding progenitors (TSPs), that arrive from bone marrow (BM) (3), develop to early thymic progenitors (ETPs) in the thymic epithelium. The ETPs in the thymus are named $CD4^-$ $CD8^-$ double-negative (DN) in stage 1. DN cells can be organized into four fractions (DN1 to DN4) (4) that are characterized by the absence of CD4 and CD8 surface expression and the differential expression of CD25, CD44, and CD117 according to development. DN1 cells ($CD44^+$ $CD25^-$ $CD117^+$) are heterogeneous and have potential to originate αβ T cells, γδ T cells, NK cells, dendritic cells, macrophages and B cells (3). The ETPs with high levels of CD117 are the most efficient at giving rise to T cells (5). Subsequently, DN1 cells leave the corticomedullary junction and migrate into the cortex towards the subcapsular zone, and differentiate into DN2 cells ($CD25^+$ $CD44^+$ $CD117^+$) (1, 6). DN2 thymocytes start to rearrange TCRβ, TCRγ and TCRδ , which are mediated by *Rag1 and Rag2* (3). When αβ and γδ T cell fate is determined, DN2 cells differentiate into DN3 ($CD25^+CD44^{lo}CD117^{lo}$) within the subcapsular zone (7). After that, DN3 thymocytes with low expression of CD27 (DN3a) give rise to DN3b, CD27 high expressing cells, which leads to β-selection. This essential checkpoint requires TCRβ chains correctly rearranged, CD3 components and an invariant pre-TCR chain

(pTα)(8). The transition to DN4 ($CD25^{-}CD44^{-}CD117^{-}$) represents the beginning of the return of cells towards the medulla (9). Initially, during the migration, CD8 is upregulated (10) (CD4 in humans), resulting in an immature intermediate single positive (SP) population, and TCRα recombination is also initiated (3). Then, cells achieve the $CD4^{+}$ and $CD8^{+}$ double positive stage (DP). DP cells undergo negative and positive selection that favors thymocytes that interact with intermediate avidity for self-peptide-MHC complexes (presented by cTECs, DCs, and fibroblasts) and express self-MHC restricted TCRs (11). The DP thymocytes then differentiate into the CD4 single positive (SP, CD4+CD8−) or CD8 SP (CD4−CD8+) lineages (12). Subsequently, SP cells migrate into the medulla and suffer a second negative selection, which exclude cells with high-affinity T-cell receptors (TCRs) for self-antigens, which avoid autoreactive T cell generation (13). One of the most important players responsible for T-cell migration is sphingosine-1-phosphate receptor 1 (S1PR1). At the end of T-cell maturation, S1PR expression increases at the cell surface, which allows cell migration from thymus to peripheral blood, where sphingosine-1- phosphate (S1P) levels are higher (14).

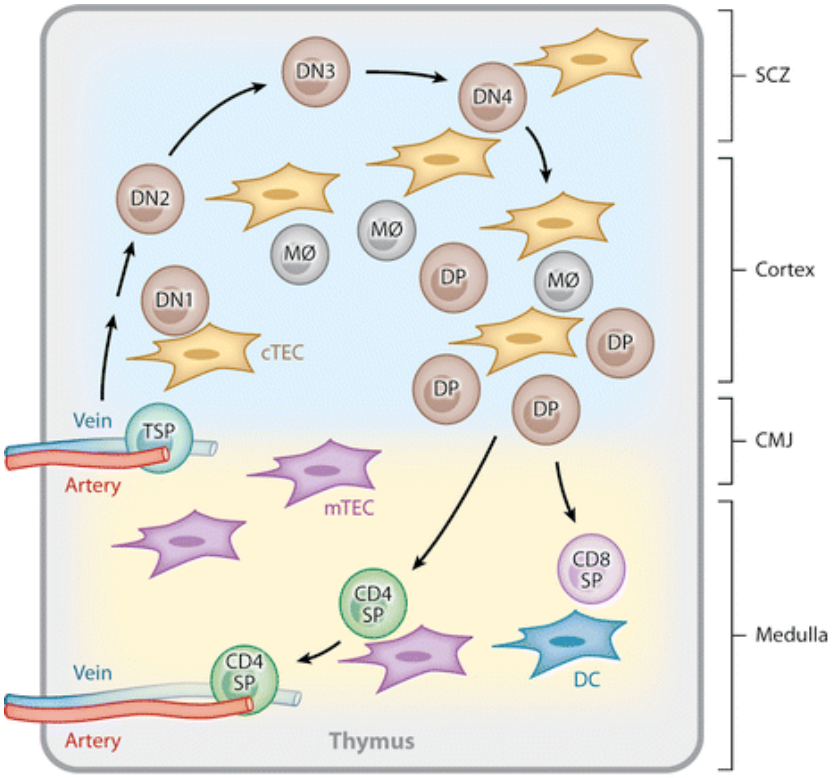

Figure 1: Schematic representation of T cell migration throughout the thymus (from Koch and Radtke. 2011).

1.2. IL-7 signaling and its importance in normal T-cell development

The IL-7 receptor is composed by an alpha subunit (IL-7Rα) (shared by the TLSP receptor) and the common gamma chain (γc), which is shared among five additional cytokine receptors (IL-2, -4, -9, -15 and -21) (15, 16). IL-7Rα is encoded by a gene on chromosome 5p13 and contains 8 exons. The cytokine anchorage to its receptor leads to receptor heterodimerization. IL-7R has no intrinsic kinase activity and so its signaling activation is dependent on two receptor-associated Janus kinases 1 and 3 (JAK1 and JAK3), which become phosphorylated and activated after IL-7 binding (17). JAK1 is associated with IL-7Rα, while JAK3 binds to γ chain (18, 19). These events precede the receptor tyrosine phosphorylation at residue 449 (Tyr449), which creates docking sites for SH2 domain proteins, such as STAT family members (20). The STAT5 and PI3K pathways, which are activated through JAK interaction, have been involved in signal transduction networks that enhance the transcription of several genes related with cell survival and proliferation of T-cells (21). These molecules could activate pro-apoptotic factors or factors that are required for cell cycle progression, for instance Bcl-2 or Cyclin D1 proteins, accordingly. On the other hand, these molecules also decrease the expression of some elements that control cell cycle progression, such as p27Kip1. (22, 23).

IL-7Rα starts to be expressed at DN2 stage, but just at DN3 stage IL-7 signaling appears to outset, simultaneously with TCR β selection (24, 25). Then, IL-7Rα expression is downregulated at DN4 until the DP stage (26). IL-7Rα shutdown is required at the end of DN stage for DP cell differentiation, enhancing TCF-1 and LEF-1 expression, which are transcription factors absolutely critical for T cell production (27). Interestingly, due to the lack of IL-7 signaling, DP thymocytes are smaller, metabolically inactive and pre-programmed to cell death, as indicated by the absence of BCL-2 and glucose transporter 1 (Glut-1) expression (26). Cell death of DP thymocytes could be rescued by survival signals, which are determined by thymic positive selection without losing self-tolerance. This means that only co-receptor tuning cells with a weak responsiveness to self-MHC receive other pro-survival signals from IL-7, excluding potential autoreactive cells (28). The TCR positive selection signals are also responsible for IL-7Rα re-expression on immature thymocytes. Additionally, IL-7Rα appears to have another crucial function, which is to instruct for CD8 T cell lineage

differentiation (29, 30). After CD8/CD4 lineage is established, thymocytes become dependent on IL-7 signaling no matter their previous choice. This requirement is just discarded with T-cell activation or memory cell differentiation when other γ chain cytokines replace its survival role (31).

Several studies have indicated that IL-7 is the most potent survival factor in T-cells and has a strong effect on thymopoiesis (32, 33). *In vivo*, using IL-7 or IL-7R blocking antibodies into wild type mice leads to severe defects on thymopoiesis, decreasing total thymocyte number, and blocking T-cell development at DN3 stage (32, 34). Furthermore, the IL-7 activity is not limited to thymocyte development: it is also responsible for the homeostasis and activity of mature T-cells (35). Lymphoid homeostasis refers to the preservation of the number and diversity of lymphocytes in an organism and this is ensured by limiting levels of IL-7 and regular contact with self-MHC (35). Given the important role that IL-7 has on regulating hematopoiesis and cellular homeostasis, its expression and production are highly regulated and site-specific. There are several transcription factors that have been associated with IL-7Rα expression, such as GA-binding protein (GABP), FoxO1, Runx 1 and Runx 3 (21, 36, 37). These molecules are responsible to control IL-7Rα transcription. In $CD8^+$ SP T-cells, the growth factor-independent-1 (Gfi-1) has been identified as a repressor of IL-7Rα transcription, which is upregulated after IL-7 signaling activation (21). Thus, downregulation of IL7Rα happens upon IL-7 signaling or T-cell activation. This allows T-cells to achieve the maximum profit of this limiting survival cytokine, in a process that is named “altruistic model” (38). The suppressor of cytokine signaling (SOCS) can inhibit IL-7 signaling induced by multiple cytokines and growth factors (39). These proteins will inhibit the STAT binding by occupying its docking site, and targeting proteins for degradation. All together, these events enable IL-7 control, which is required to escape from pathway saturation, and this fine-tuning of the signal is essential to achieve the molecular balance for cell survival. IL-7Rα downregulation is also critical for normal T-cell development, allowing to select the immature thymocytes that correctly rearrange a functional and useful T-cell receptor (TCR). Autoimmune and chronic inflammatory diseases, and lymphoproliferative disorders have been correlated with abnormal IL-7 signaling levels (40, 41).

1.3. T-cell Acute Lymphoblastic Leukemia (T-ALL)

Acute lymphoblastic leukemia (ALL) is the most common cancer in pediatric patients, which has been organized into precursor T (or T-cell), precursor B, and B-cell (Burkitt) phenotypes. These groups can be sub-characterized according to recurrent karyotypic abnormalities.

T-cell acute lymphoblastic leukaemia (T-ALL) is an aggressive malignancy that comes up during thymocyte development, which leads to an arrest at early stages of T-cell maturation. This disease comprises 15% of pediatric and 25% of adults' acute lymphoblastic leukemia events. This pathology is clinically characterized through the extension of bone marrow involvement, normally with 25% or more blasts present in the BM, and also with the presence of blasts in the peripheral blood (42, 43). Regarding its clinical features, T-ALL frequently presents splenomegaly, adenopathy, mediastinal lymph nodes and thrombocytopenia (44). Despite BM being the predominant site of this disease, this malignancy tends to disseminate to all over the body, and could even affect the central nervous system (45, 46).

Different genetic abnormalities in TCR gene loci have been linked to T-cell transformation in T-ALL development. These alterations are promoted by chromosomal translocations that lead to the juxtaposition of genes encoding transcriptional regulators close to TCR gene enhancers and promoters, which drive its expression. This comprises genes such as MYC, TAL1, LYL1, LMO2, TLX1, TLX3 HOXA and MYC (Figure 2) (47). These genetic alterations are used to classify T-ALL into subgroups. Other genetic abnormalities have also been described, for instance mutations and non-random chromosomal translocations driving constitutive activation of NOTCH1, which altogether are reported in 31-60% of T-ALL patients (48).

Nowadays, T-ALL treatment regimens include a combination of up to 10 different drugs, such as L-asparaginase, vincristine, prednisone, cyclophosphamide, doxorubicin, cytarabine, cyclophosphamide (49), normally composed by an intensive induction followed by consolidation and continuation therapy phases (49). Due to the fact that patients tend to suffer of central nervous system involvement, T-ALL patients normally receive a prophylaxis treatment for central nervous system with triple intrathecal medications (methrotrexate, cytosine arabinoside and hydrocortisone) (49).

There are some variables that influence patient's prognosis and have been used to determine high risk patients, such as age, race, white blood cell counts (WBC), hemoglobin, and presence of a mediastinal mass or extramedullary disease (50). Regarding the age at diagnosis, children with < 1 year and ≥ 10 years have worse prognosis. In these higher risk ages, there is an inverse correlation between age and prognosis (50, 51). Males and blacks have a slightly worse prognosis (52). Different studies have shown that WBC counts (≥ 50 x 10^9/l) and central nervous system involvement have also worse prognosis (50). Maturational stage of the dominant clone has been suggested to predict patient's outcome, with some evidence, although controversial, that more mature stages, as cortical T-ALL, have better prognosis.

Children with T-ALL have worse prognosis comparing with B-ALL, with a higher number of high-risk patients and a higher tendency to have treatment resistance. Despite the comparable outcomes for T- and B-ALL high-risk patients, relapsed T-ALL have a disastrous prognosis with 3-year rates of event-free survival <15% (53). Over the past years, patient's outcome has been improved through more efficient tools for risk stratification, more aggressive chemotherapies and stem cell transplantation to overall survival rate of 70% for children and 30-40% for adults below 60 years and 10% above 60 years. Despite these progresses, the high incidence of relapses remains a huge problem in T-ALL.

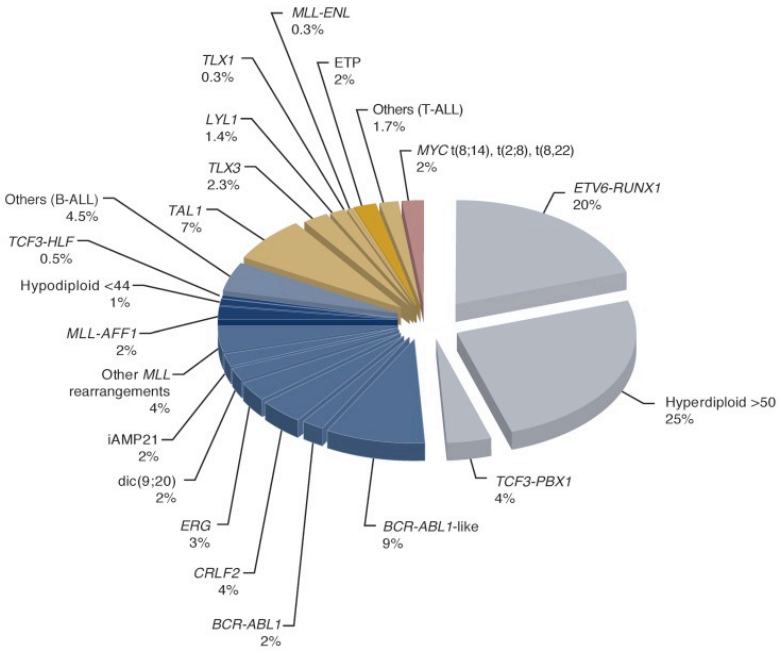

Figure 2: **Frequency of cytogenetic subtypes of childhood ALL.** It shows the relative frequencies of T-ALL (yellow) genetic subtypes. This pie chart does not include submicroscopic genetic alterations. Data from Pui et al (2012).

1.4. IL-7 signaling in T-ALL development

Considering that IL-7 signaling is critical for normal T cell development and cell survival, it is acceptable to hypothesize that survival of malignant T cells could be supported by IL-7. It has also been shown that pathological constitutive activation of IL-7 pathway enhances T-ALL progression, which has been associated with its leukemogenic potential (54).

AS described previously, the altered responses of leukemic cells underlie critical changes in gene expression. One of the most frequent mutated genes in T-ALL is NOTCH1, whose mutations are present in more than 50% of T-ALL patients, as already mentioned. IL7Rα is a well-known direct target of NOTCH1, hence its upregulation in T-ALL with mutated NOTCH1 (48). Overexpression of IL-7 is also observed in thymocytes from AKR/J mice, which are highly susceptible to develop spontaneously thymic lymphomas (55). Besides this, several studies have demonstrated that a majority of T-ALL patients respond to IL-7 *in vitro* and IL-7 participates in disease expansion in xenotransplantation models of human T-ALL (56, 57). Taken together, these evidences suggest that deregulation of IL7-mediated signaling has a central role in T-ALL development.

Furthermore, there are several genetic hints that indirectly discloses the importance of IL-7 in T-ALL, such as activating mutations in JAK1. These gain-of-function mutations are present in 10-20% of T-ALL, and enhance constitutive activation of STATs, AKT and ERK proteins that are downstream of IL-7Rα (58). Additionally, JAK3 gain-of-function mutations have also been described in 10% of a subgroup of high-risk (T-ALL) patients T-ALLs characterized by Early T cell phenotype (ETP-ALL) (59). Altogether, these studies indicate that JAK1 and JAK3 mutations have transforming potential in T-ALL.

Recently, it has been shown that gain-of-function mutations in IL-7Rα are present in 10% of T-ALL patients. IL-7Rα mutants induce constitutive signaling independently not only of its ligand but also of γc and JAK3. In most cases, these mutations give rise to intermolecular disulfide bonds, which result from the insertion of an unpaired cysteine in the juxtamembrane region of IL-7Rα, a mutational hotspot region encoded by exon 6, leading to receptor homodimerization. This event triggers JAK/STAT5 and PI3K/Akt/mTOR pathways. Murine B-cell line (Ba/F3) transduced with mutant hIL-7Rα

showed that the mutants promote both cell cycle progression and viability in the absence of growth factors (54). These findings gave a new perspective of IL-7/IL-7R-mediated signaling in T-ALL, which could potentiate new therapeutic strategies.

As previously described, IL-7 signaling leads to the activation of JAK/STAT and PI3K/Akt/mTOR pathways that enhance the transcription of several genes related with cell survival and proliferation (60). JAK/STAT5 is required for IL-7-dependent T-cell lymphomagenesis in mice (61), however its role in human T-ALL remains to be elucidated. PI3K activates downstream factors such as Akt and its downstream target mTOR. Genetic alterations in this pathway (including defects in its major negative regulator, the lipid phosphatase PTEN) affect 48% of T-ALL cases (62). However, hyperactivation of PI3K/Akt pathway is even more frequent in T-ALL due to posttranslational alterations, which contribute to leukemia cell maintenance (60). Notably, IL-7 is able to upregulate the activity of this pathway even further and T-ALL cells, in contrast to healthy T cell precursors, activate PI3K/Akt/mTOR pathway upon IL-7 stimulation in a manner that is responsible not only for cell cycle progression, but also for Bcl-2 upregulation and the consequent increase in cell viability (63, 64).

These findings demonstrate an oncogenic role of the IL-7 receptor in T-ALL and suggest, perhaps, that the majority of T-ALL subtypes harbors an activated IL-7R pathway, suggesting the IL-7Rα as a potential therapeutic target in this malignancy.

1.5. Sphingolipid Rheostat

Sphingolipid metabolites have been identified as signaling players with a meaningful influence in cell fate, leading to cell proliferation/ survival or apoptosis. Therefore, sphingolipid synthesis and degradation pathways are highly regulated processes, which are determined by different players. Ceramide can be generated by *de novo* synthesis, breakdown of sphingomyelin or complex glycosphingolipids, and then can be deacylated into sphingosine. A key molecule in these pathways is sphingosine kinase (SPHK), which is responsible for the conversion of sphingosine into sphingosine-1-phosphate (S1P). The levels of these molecules have a role in the balance between cell apoptosis (sphingosine) or cell survival (S1P). This important switch, named sphingolipid rheostat, has been related with survival, proliferation, migration, angiogenesis, inflammation and differentiation (65). The biologically active phospholipid S1P can be reversibly modified into sphingosine by S1P phosphatases

(SPP1 and SPP2), or cleaved irreversibly into hexadecenal and phosphoetanolamide by S1P lyase (SPL) (66, 67).

Commonly, S1P is generated intracellularly and then exported by ATP-binding cassette (ABC) super family of transporters or other mechanisms (68). S1P in the circulation can bind to a family of five S1P-specific cell surface G protein-coupled receptors (GPCRs) (S1PR1 to S1PR5) and enhances different signaling pathways, an event that is named inside-out signaling. For instance, PI3K/AKT, ERK1/2 or PLC are some of those S1P-dependent activation signaling pathways that are responsible for cell proliferation, migration, survival, angiogenesis, maturation, inflammation, immunity, chemotaxis and trafficking (65). S1P functions are not exclusively extracellular, since S1P also modulates histone deacetylases leading to c-fos, cell cycle regulator CDKN1A (p21) expression (69) and the ubiquitin ligase activity of tumor necrosis factor receptor-associated factor 2 (TRAF2) (70). S1P-induced TRAF2 activity and NF-kB activity afterwards have a pro-survival/pro-proliferative effect.

1.6. S1P in normal hematopoiesis

S1P has been described as one of the major players in lymphocyte movement between BM and secondary lymphoid tissues into peripheral blood. S1P differential gradient between the lymphoid organs and the periphery allows lymphocyte egress. The first evidence of this function came from the discovery that the sphingosine analogue and SPHK inhibitor, Fingolimod (FTY720), leads to rapid but reversible lymphopenia, and lymphocytes are retained at secondary lymphoid organs simultaneously (14, 71). These studies also showed that S1P leads to rapid receptor internalization and degradation and this leads to a decrease in cell response. The same effect that occurs in mature lymphocytes happens in thymocytes (72). At the end of thymic maturation, S1PR overexpression is required to enable the exit from the thymus. The role of S1P in lymphocyte migration is also involved in inflammatory processes (73). The injured tissues increase their S1P levels, which promotes lymphocyte retention through S1PR1. These findings altogether demonstrate that S1P has an important role in hematopoiesis, however further experiments are required to clarify its function.

1.7. The Sphingosine Kinases: differences and functions

There are two human SPHK genes, *SPHK1 and SPHK2*, which are located on chromosome 17 (17q25.2) and 19 (19q13.2) respectively, and encode SPHK1 and SPHK2 proteins. These are highly conserved lipid kinases which are responsible for S1P synthesis (74). Despite their high polypeptide sequence similarity (80%), sharing five highly conserved regions (C1-C5), SPHK2 presents two extra polypeptide regions at the N-Terminus and a central proline-rich region (75). Their differences are not only restricted to their structure. In fact, SPHKs display distinct development expression, adult tissue distribution, substrate binding, and subcellular localization, which could foresee distinct physiological functions (76). Although SPHK isoforms are expressed in all tissues in humans, SPHK1 is highest expressed in lung, spleen and leukocytes (77), whereas SPHK2 is increased in the kidney and liver. In addition, SPHKs are also differentially expressed in embryonic development (78). In mouse, SPHK1 expression increases earlier (E7-E11), while SPHK2 expression raises later (E15-E17) in embryogenesis (78).

Different studies show that SPHK1 and SPHK2 may have antagonistic roles in diverse pathological conditions, such as inflammatory arthritis, kidney injury, lipopolysaccharide-induced injury and cancer. In the majority of the reports, SPHK1 enhances cell survival and proliferation in different types of cancer (79-81), while there is no agreement respecting to SPHK2 functions. Two contrasting roles, pro-apoptotic and anti-apoptotic functions are suggested and SPHK subcellular position seems to be critical in this choice.

Despite their different roles, genetic deletion of SPHK1 or SPHK2 has no effect in normal development in mice, and deletion of both SPHKs results in mice lifeless *in utero* due to several abnormalities in angiogenesis and neurogenesis (82). Altogether, these data indicate that SPHKs have some functional redundancy, at least in normal mouse development.

1.8. Sphingosine kinase 1

There are three different SPHK1 isoforms (SPHK1a, SPHK1b, and SPHK1c) that diverge in their N-terminal domain (75). SPHK1a is secreted by human umbilical-vein endothelial cells and seems to contribute to increased S1P plasma levels (83).

SPHK1b is located at the cell membrane (84), while SPHK1c is at the Golgi and is present in lower levels comparing with its homologous (83). Despite these differences, all of them have intrinsic catalytic activity, and can be additionally activated by growth factors (EGF, PDGF, NGF, VEGF), cross-linking of immunoglobulin receptors, TGFB and TNFα, adhesion molecules (PECAM1) (85), interleukins, Ca^{2+} increasing agents and phorbol esters (74). Whereas some of these molecules lead to a rapid and transient activation of SPHK, promoting posttranslational modifications or changing its intracellular localization, others induce a biphasic mechanism, increasing initially its activity and then its transcription, such as EGF, estrogen, 1,25-dihydroxyvitamin D3 or histamine.

Activation of SPHK1 was shown to be mediated by protein kinase C, classical MAPK/extracellular-signal-regulated kinases (ERKs) and PI3K/mTOR and promotes phosphorylation of SHPK1 at Ser^{225} by ERK1/2 (86). The phosphorylation of SPHK1 increases its selectivity for phosphatidylserine at the inner leaflet of the plasma membrane via the side chains Thr^{54} and Asn^{89}. This transient activation could be countered by protein phosphatase 2A (PP2A) (87). In this model, phosphorylation of Ser^{225} promotes a conformational alteration that exposes Thr^{54} and Asn^{89} and/or other phosphatidylserine-binding sites, which helps membrane translocation (88). Enzymatic activity is explained by the proximity of SPHK1 to its substrate, sphingosine, and by the stimulatory action of phosphatidilserine (88). Some recent evidences show that calcium and integrin-binding protein 1 (CIB1) facilitates active translocation of SPHK1 to the plasma membrane in a calcium-myristoyl dependent way and it is maintained at this localization through acidic phospholipids and other interactions (89).

1.9. Sphingosine kinase 2

SPHK2 presents two isoforms, named SPHK2-S and SPHK2-L. The last mentioned isoform contains 36 additional amino acids and is the most common in humans (83).

SPHK2 can also be activated by different factors, such as EGF (90), TNF-α (91), cross-linking of the IgE receptor FcεRI (92), phorbol esters (90) and hypoxia conditions (93). This lipid kinase, similarly to SPHK1, can also be activated by ERK1/2 by phosphorylation at Ser^{351} and Thr^{578} (94).

SPHK2 displays nuclear localization (NLS) and export (NES) signals (95, 96). When SPHK2 is located in the nucleus, it prevents DNA synthesis (96) and regulates epigenetic modifications modulating HDAC1/2 (97). Increased acetylation of H3, H4 and H2B leads to increased expression of cyclin-dependent kinase inhibitor (CDKN1A) and the transcriptional regulator FOS (69). Besides this, SPHK2 localized at the nucleus seems to be related with contact inhibition responses, which decrease cell proliferation. On the other hand, it has been shown that SPHK2 is located at endoplasmic reticulum under stress conditions, which is associated with increased levels of pro-apoptotic ceramide (76). SPHK2 positioning at mitochondrial membrane also leads to cell apoptosis via S1P, BAK and cytochrome c release (97). By contrast, SPHK2 placed at the plasma membrane promotes cell proliferation. Interestingly, it has been shown that SPHK1 artificially targeted to the ER or nucleus leads to apoptosis (76). These findings indicate the major involvement of the SPHK location in its functions.

1.10. Sphingosine kinase role in malignant development

Different studies have demonstrated that SPHK1 displays oncogenic features. Normal NIH3T3 fibroblasts that have upregulated SPHK1 show tumorigenic features, as indicated by colony growth in soft agar (98).

Overexpression of SPHK1 has been shown in different types of cancer, such as breast, lung, ovary, stomach, uterus, kidney, where its levels are 2-fold higher comparing with normal tissue (99, 100). Furthermore, SPHK1 upregulation has also been associated with poor prognosis in gastric, breast, brain and lung cancer patients (81, 101, 102). Moreover, SPHK inhibitors significantly decrease tumor growth in mice (103, 104) and cells lacking SPHK1 become more sensitive to chemotherapeutic treatments (105).

Several studies have demonstrated the proliferation potential of SPHK1 in hematological malignancies. SPHK1 activation is promoted by interleukin-6 in multiple myeloma cells (106), and PDGF seems to induce a similar effect in T-cell large granular lymphocytic leukemia (T-LGL) (107). Interesting, protein kinase C (PKC), classical MAPK/ ERKs and PI3K/mTOR are some of the signaling pathways that are implicated in SPHK1 activation and which have been shown to be deregulated in leukemia (86). In erythroleukemia, SPHK1 overexpression is required to promote

leukemia development and progression (108), and BCR/ABL1 increased SPHK1 expression in CML (109). Furthermore, S1P pro-survival signals avoid cytochrome c and Smac/Diablo release in leukemia cell lines, upregulating anti-apoptotic molecules such as Mcl-1 in chronic myeloid leukemia and multiple myelomas (110). Inhibition of SPHK1 activity in leukemia cells, for instance in CML (111) and T-LGL (112), promotes cell death.

As described before, while SPHK1 role in cancer is well established, SPHK2 appears to have a dual role in cancer development and progression. Some studies have demonstrated that SPHK2 has also a similar function to SPHK1. In MCF7 human breast cancer cells, SPHK2 knockdown prevents growth of xenografted tumors (113). Further, some studies showed that chemical inhibition of SPHK2 reduced proliferation and induced autophagy cell death in kidney, prostate and breast cell lines (114, 115).

Although the effects of SPHK2 in hematological malignancies are much less known, the importance of SPHK2 starts to emerge. Recently, Wallington-Beddoe et *al.* showed that SPHK2 has a role in B-ALL, through the expression of the oncogene MYC and that SPHK2 activity signature is increased in ALL patients (116).

Together, these studies elucidate the critical involvement of SPHK in malignant development, and consequently SPHK arises as an evident therapeutic target. There are several pre-clinical studies using FTY720 inhibitor in disseminated lymphoma (117), CML (118), murine cell line models of AML with KIT mutations (119), and also in rat models of NK leukemia (119). Moreover, in mice engrafted with human AML U937 cell line, treatment with a SPHK1 inhibitor reduces tumor growth (120), and in a human xenograft model of Ph+ ALL, a SPHK2 inhibitor has anti-leukemic potential, which cooperates with Imatinib (116). Nonetheless, the effects of SPHK1 and SPHK2 inhibitors on hematological malignancies are still poorly explored.

OBJECTIVES

Taken all of the findings summarized above together, we hypothesized that SPHK1 and/or 2 (hereby jointly reffered to as SPHK) may be involved in T-ALL and particularly in IL-7-mediated signaling in this malignancy. Hence, this research project intended to investigate the importance of SPHK in normal and mutant IL-7Rα signaling in T-ALL.

To accomplish our main goal, we defined some critical questions:

1. What are the levels of SPHK expression? Are they increased in T-ALL patients?
2. Does IL-7 regulate SPHK expression and/or activity?
3. Is SPHK involved in IL-7 mediated signaling in T-ALL cells?
4. What are the effects of SPHK inhibition on IL-7-mediated cell viability, cell cycle, activity and proliferation of T-ALL cells?

In order to address these questions, we used the following cell lines: D1, a murine IL-7-dependent thymocyte-like cell line; TAIL7, a human IL-7-dependent primary-like T-ALL cell line; DND4.1, a human IL-7Rα mutant T-ALL cell line. These cell lines were cultured with a SPHK pan inhibitor, SKI-II. Although we performed some analyses discriminating between SPHK1 and 2, the functional studies we performed so far made use only of SKI-II. As such, we opted to name SPHK1 and SPHK2 jointly as SPHK in most parts of the present thesis, although we are aware that not necessarily the two are involved in IL-7-mediated signaling.

We speculated that SPHK may partake in and drive IL-7-mediated signaling pathways, by being required for full activation of IL-7 downstream elements (Figure 3). If confirmed, this could constitute the first step towards the characterization of SPHK as a key molecule in IL-7-mediated signaling and a new potential therapeutic target in T-ALL.

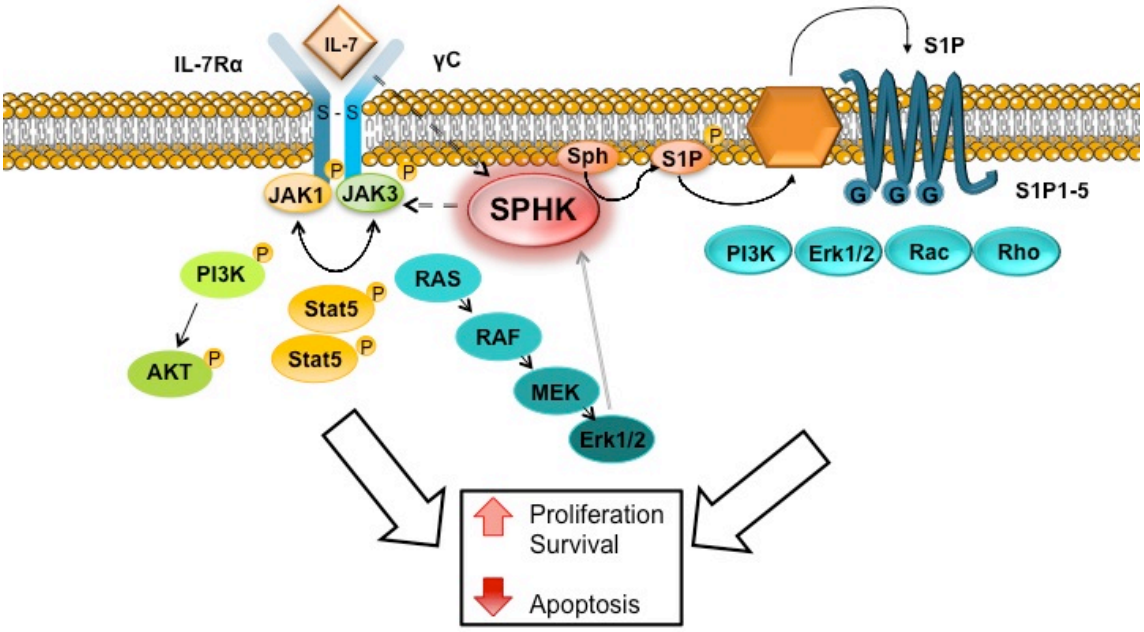

Figure 3: **Proposed model for SPHK role in IL-7-mediated signaling.** The dashed arrows represent our hypothesis, as SPHK could partake in IL-7-mediated signaling pathways by being required to the full activation of IL-7 downstream elements.

MATERIALS AND METHODS

3.1. Reagents and Antibodies

SKI-II, 2-(p-Hydroxyanilino)-4-(p-chlorophenyl) thiazole, which is a SPHK pan-inhibitor, was synthesized by Calbiochem® (Merck Millipore).

The following were used as primary antibodies: p27kip1 (BD Transduction Laboratories), phospho-JAK3 (Y980), JAK3, JAK1, STAT5, SPHK1, Actin, Caspase-3, (Santa Cruz Biotechnology), phospho-STAT5A/B (Y694/Y699; Upstate Biotechnology), and phospho-JAK1 (Y1022/1023), phospho-Akt (S473), phospho-Erk1/2 (T202/Y204), Akt, Erk1/2, phospho-PTEN (S380) and PTEN (Cell Signaling Technology). Immunodetection was performed with horseradish peroxidase–conjugated anti–mouse IgG, anti–rabbit IgG or anti–goat IgG (Promega).

3.2. Cell Culture

TAIL7, a human T-ALL IL-7 dependent primary-like cell line, was grown in the presence of 10ng/ml of human IL-7, in RPMI medium (Gibco) supplemented with 5% of fetal bovine serum (FBS) (PAA Laboratories GmbH), at a concentration of 2 × 10^6 cells/ml. The human T-ALL cell line DND-4.1 was cultured in RPMI 10% of FBS (Biowest) at a concentration of 0,5× 10^6 cells/ml. The murine IL-7 dependent cell line D1 was grown in the presence of 50 ng/ml of human IL-7 in RPMI with 10% of FBS (PAA), at a concentration of 1 × 10^6 cells/ml. The human T-ALL cell line Jurkat was cultured in RPMI 10% of FBS (PAA) at 0,5× 10^6 cells/ml.

3.3. *In Vitro* Culture

TAIL7 cells were plated in 6-well plates or 96-well plates at 2×10^6 cells/ml at 37°C with 5% CO_2. Serum and IL-7 starved TAIL7 cells for 24h were incubated in RPMI 5% FBS (control medium) with 20 ng/ml of IL-7, SKI-II or both. Following, cells were harvested and processed for western blot or FACS analysis, accordingly.

D1 cells were plated in 6-well plates or 96-well plates at 1×10^6 cells/ml at 37°C with 5% CO_2 in RPMI 10% FBS (control medium) in the presence of 50 ng/ml IL-7 and SKI-II, or both. D1 cells were starved in RPMI without FBS and IL-7 for 2h prior to any stimulation.

DND-4.1 cells were plated in 6-well plates or 96-well plates at $0,5\times10^6$ cells/ml at 37°C with 5% CO_2 in RPMI 10% FBS (control medium) with or without SKI-II.

3.4. Protein Extraction

Collected cells were centrifuged at 3500 rpm for 5 minutes at 4ºC in order to obtain a cell pellet. Cell pellet was ressuspended in lysis buffer (50mM Tris-Base; 150mM NaCl; 5mM EDTA; 1mM NaOVa; 10mM NaF; 10mM NaPyrophosphate; 1% NP-40, supplemented with protease inhibitor cocktail Complete Mini (Roche) and protease inhibitor AEBSF (1mM)). The extracts were centrifuged at 13000 rpm for 15 minutes at 4°C and the supernatants were collected. Total protein quantification was performed by Bradford assay (absorbance at 595nm). Protein extracts were ressuspended in Laemmli buffer and denatured at 95 °C for 5 minutes.

3.5. Western Blot

Samples were separated by SDS-PAGE (12 or 14% of acrylamide gel) and transferred onto a nitrocellulose membrane. Precision Plus Protein™ (Bio-Rad) molecular weight standards were used as reference. Protein transference was carried out using conventional transference system (90 min, 400mA). Membranes were submerged in Ponceau S solution in order to evaluate the protein content. The membranes were then blocked (3% skimmed milk, TBS-T buffer) and then incubated with primary antibody diluted in TBS-T (in pre-established dilutions and antibody specific) overnight at 4°C under constant agitation. After washing the membranes for 25 min in TBS-T (in a total of three washing steps), the membranes were incubated with the respective secondary antibody diluted (1:5000) in a 3% milk/TBS-T buffer blocking solution and incubated for 1 hour at room temperature under constant agitation. Chemiluminescence detection was carried out using the Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Sientific Inc.). Membranes were revealed using the Curix60 (AGFA HealthCare).

3.6. Membrane Stripping

In order to perform a new blot of the membrane, the antibodies were removed by stripping process. The membranes were incubated in the stripping solution (Tris-HCl,

β-mercaptoethanol (100mM), 10% SDS, pH 6.7) for 30 minutes with mild agitation at 56ºC. Finally, the membrane was washed in water and then in TBS-T. Primary and secondary antibody solutions were then applied as described before.

3.7. Quantitative Real-Time PCR

The mRNA expression of SPHK1 and 2 was quantified using a 7500 Fast or ViiA 7 Real-Time PCR System (Applied Biosystems). The following primer sequences were used, human SPHK1 forward: CTT GCA GCT CTT CCG GAG TC and reverse: CAC GCT GAT GCT CAC TGA GC; mouse SPHK1 forward: TGT GAA CCA CTA TGC TGG GTA and reverse: CAG CCC AGA AGC AGT GTG, 18 S rRNA forward: GGA GAG GGA GCC TGA GAA ACG and reverse: CGC GGC TGC TGG CAC CAG ACT T, human SPHK2 forward: CTG TCT GCT CCG AGG ACT GC and reverse: GAC CCC CAA AGG GAT TGA CCA AT; mouse SPHK2 forward: GGA GACG GGC TGC TTT ACG AG and reverse: (tudo o que está sublinhado não está a preto, está a cinza. Confere) GATCCACAGGGGAGGACACC.

3.8. Analysis of cell viability, size and CD71 surface expression

Cell viability was determined by double-staining with APC-conjugated Annexin V (eBioscience) and 7-AAD (BD Biosciences) followed by flow cytometric analysis on a LSRFortessa flow cytometer (BD Biosciences). Briefly, cells were washed with PBS and ressuspended in 100 µL of binding buffer with Annexin V and 7-AAD. After 15 minutes of incubation at room temperature in the dark, 100 µL of binding buffer were added and the samples were acquired. Fully viable cells were identified as the Annexin V and 7-AAD double-negative populations.

Cell size was assessed by analysis of SSC versus FSC flow cytometry plots gated on the live cell population. Samples were analyzed using LSRFortessa flow cytometer (BD Biosciences) and FACSDiva software (BD Biosciences). Results were analyzed by FlowJo software (Tree Star).

Transferrin receptor (CD71) surface expression was analyzed by flow cytometry using FITC-conjugated anti-CD71 (eBioscience) antibody. Results were expressed as the specific mean intensity of fluorescence (MIF).

3.9. Cell Cycle Analysis

Cells were harvested, washed with PBS and fixed in 80% ethanol at -20ºC. Cells were ressuspended in PBS containing 50 ug/ml RNase A and incubated at 37ºC for 30 minutes. Samples were labeled with 100ul/ml propidium iodide (PI) and were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Cell cycle modeling was performed using Modfit^{TM} LT software (Verity Software House Biosciences).

3.10. Assessment of Mitochondrial Membrane Potential ($_{\Delta}\psi_m$)

Cells were harvested and stained in culture medium with TMRE (Sigma-Aldrich) to a final concentration of 100 nM, and incubated for 15 min at 37°C. Cells were analyzed for TMRE intensity by flow cytometry. Samples were analyzed using LSRFortessa flow cytometer (BD Biosciences) and FACSDiva software (BD Biosciences). Results were analyzed by FlowJo software (Tree Star).

3.11. Proliferation Assays

Cells were cultured in triplicates in flat bottom 96-well plates at the normal cell culture density and conditions as indicated previously. IL-7 cytokine and/or SPHK inhibitor were added in the experimental conditions mentioned above. Cells were incubated with [^{3}H] labelled thymidine at the concentration of 1μCi/well for 16 h (TAIL7 cell line) or 8h (remaining cell lines) before harvest. DNA synthesis, as measured by [^{3}H] thymidine incorporation, was assessed using a scintillation counter (Perkin Elmer). Average and standard deviation of triplicates were calculated.

3.12. Sphingosine Kinase Activity Assay

The SPHK Activity was measured through a commercial kit from Echelon Inc. (SPHK Activity Assay). This is a based luminescent assay that measures SPHK activity by detecting the consumption of ATP transformation of sphingosine into S1P. The experimental conditions were conducted as described previously. Total cell lysates were collected in lysis buffer (described previously in western blot section) and supplemented with Triton-X 100 (0,1%) or KCL (1M) in order to discriminate SPHK1 and SPHK2 activity, respectively. The assay was performed accordingly to

manufacturer instructions. The luminescence signal was detected after one hour of incubation with the microplate reader Infinite M20 (Tecan). The integration time was set to 1000ms per well.

3.13. Statistical analysis

Statistical computations were performed with GraphPad Prism version 5.0 for Windows (GraphPad Software). In what concerns the differences among groups, it was performed ANOVA, t-Student or Mann-Whitney statistical tests, as appropriate. Differences among groups were considered statistically significant at $p \leq 0,05$ (95% confidence interval).

RESULTS

4.1. Sphingosine Kinase Expression in T-ALL

Given the existing evidence that SPHK is upregulated in other leukemias, we analyzed gene expression of SPHK1 and SPHK2 in T-ALL patients and normal thymocytes by qRT-PCR. SPHK1, but not SPHK2, transcript levels were increased in T-ALL samples ($p = 0.0462$) comparing with normal thymocytes (Figure 4A). We also analysed a publicly available dataset (GSE7186) for SPHK expression, that corroborated our initial analysis and showed that SPHK1 expression was higher in T-ALL ($p = 0.0439$) as compared to normal bone marrow samples (Figure 4A and 4B).

In order to determine the effect of IL-7 stimulation on SPHK1 gene expression, we incubated TAIL7 and D1 cell lines in the presence or absence of IL-7. We found that IL-7 did not significantly affect SPHK1 expression (Figure 4C). Similar results were obtained for SPHK2 expression with or without IL-7 stimulation (data not shown). The effect of IL-7 on SPHK activity was then evaluated in TAIL7 and D1 cell lines. In contrast to the expression levels, IL-7 stimulation in TAIL7 cells clearly upregulated total SPHK activity ($p = 0.0393$, Figure 4D). In D1 cells, we observed that SPHK1 activity was higher than SPHK2 activity and that IL-7 withdrawal decreased the activity of both enzymes ($p = 0.0305$ and $p = 0.0042$, Figure 4D).

Together, these results show that SPHK1 is significantly more expressed in T-ALL samples compared to their normal counterparts and that IL-7 stimulation positively regulates SPHK kinase activity without significantly affecting SPHK transcript levels.

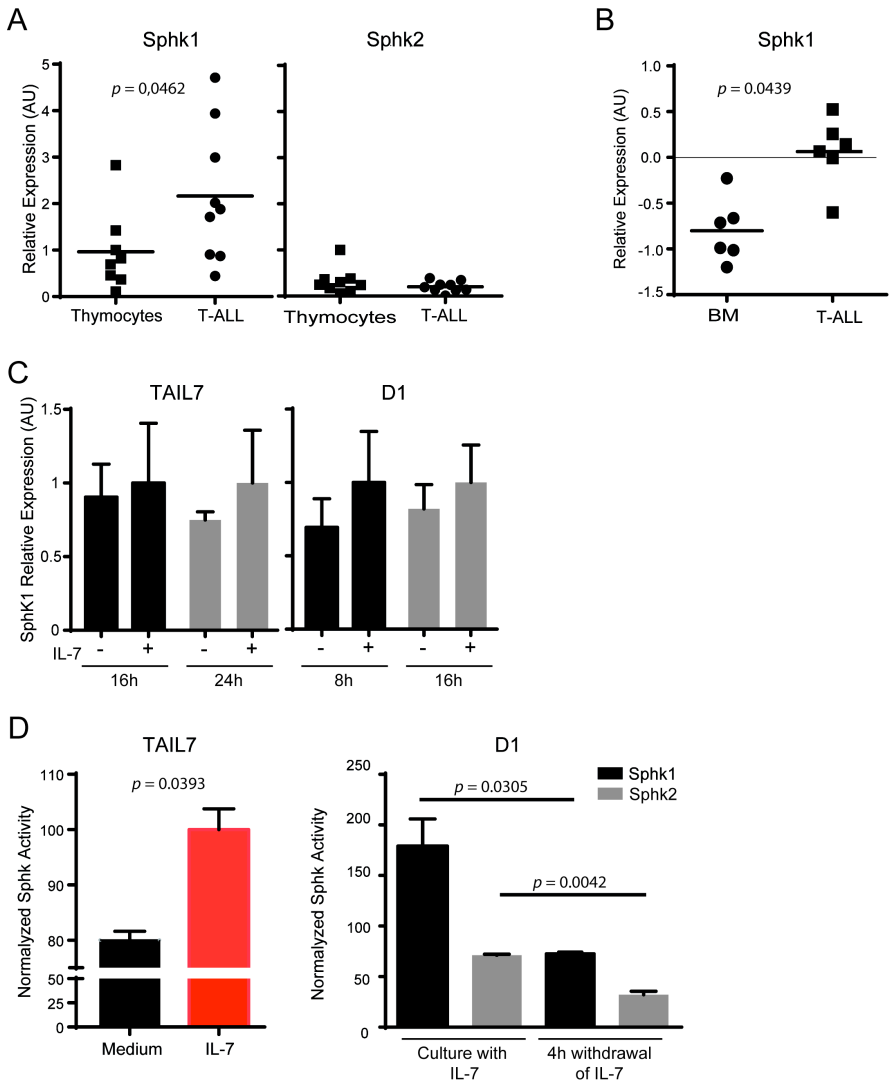

Figure 4: IL-7 stimulation positively regulates SPHK mRNA levels and activity. (A) Peripheral blood or bone marrow samples were collected from T-ALL patients at the time of diagnosis and total RNA was extracted. Statistical analysis was performed using Mann-Whitney test from triplicates. Relative expression of *SPHK1* was significantly different between groups (p = 0.0462), whereas no statistical differences in *SPHK2* expression were observed. (B) A publicly available dataset of gene expression (GSE7186) was manual analysed. Statistical analysis was performed using Mann-Whitney test (p = 0.0439). (C) TAIL7 cells were starved for 24h and cells were collected after 16 and 24 hours of incubation in the presence or absence of 20ng/ml of IL-7. D1 cells were starved for 2h and cells were collected after 8 and 16 hours of incubation with presence or absence of 50 ng/ml of IL-7. Then RNA was extracted with subsequent cDNA production and qRT-PCR analysis. The observed differences between IL-7 presence and absence were not statistically significant. (D) TAIL7 cells were starved for 24h and protein extracts were collected after 24 hours of incubation in the presence or absence of 20ng/ml of IL-7. D1 cells protein extracts were collected after 4 hours of IL-7 withdrawal. Then total SPHK, SPHK1 and SPHK2 activities were measured with specific buffers by commercially available kinase activity assay. Statistical analysis was performed using 1-Way ANOVA from duplicates, p = 0.0393 in TAIL7 cells. In D1 cells was performed a *t*-Test from duplicate values, p = 0.0305 for SPHK1 conditions and p = 0.0042 between SPHK2 conditions.

4.2. Sphingosine Kinase inhibition effect in IL-7 mediated-signaling

To determine the role of SPHK in IL-7-mediated signaling in normal and T-ALL cells, we used different cell lines. In particular, D1 ('normal', non-tumorigenic thymocyte IL-7-dependent murine cells), TAIL7 (T-ALL cells, IL-7-dependent) and DND-4.1 (T-ALL cells, IL-7Rα mutant) were treated with the pan-SPHK inhibitor SKI-II. D1 and TAIL7 cells were starved to potentiate the responsiveness to IL-7 and then treated with SKI-II. The inhibitor blocked IL-7-mediated upregulation of STAT5, AKT and S6 phosphorylation without affecting total protein levels (Figure 5A). These effects on IL-7-mediated signaling were present in both normal and T-ALL contexts, *i.e.* in D1 and TAIL7 cells, respectively (Figure 5A).

To determine the impact of SPHK inhibition on IL-7Rα gain-of-function mutations, we used the DND-4.1 cell line, which has a heterozygous 12 nucleotide insertion in the IL-7R gene (IL7R p.L242_L243insLSRC).This alteration is similar to the mutations that were described in 10% of T-ALL patients, which promote constitutive activation of IL-7R without IL-7 requirement. Western blot analysis of DND-4.1 cells corroborated the constitutive phosphorylation of IL-7R downstream targets, such as STAT5 and AKT (Figure 5A). SKI-II treatment significantly downregulated STAT5 and AKT phosphorylation levels and had a perceptible, although minor, effect on S6 phosphorylation (Figure 5A).

In order to identify the proximal targets of SPHK in IL-7 signaling, we next conducted a time course experiment with SKI-II pre-incubation for two hours with subsequent IL-7 stimulation. The early responses to IL-7 stimulation in the presence of SPHK inhibition were analysed by western blot. Our data demonstrate that SPHK activity is required for very early STAT5 and JAK-1 phosphorylation (30 seconds post-stimulation), as well as for JAK3 early phosphorylation (1 minute after IL-7 stimulation) (Figure 5B). Phosphorylation of downstream effectors, such as AKT and S6, was also affected. In contrast, PTEN phosphorylation, which is not IL-7-dependent, was not affected by SKI-II (Figure 5B).

These data suggest that somehow SPHK activity is required for optimal triggering of IL-7-mediated signaling in both normal and pathological contexts.

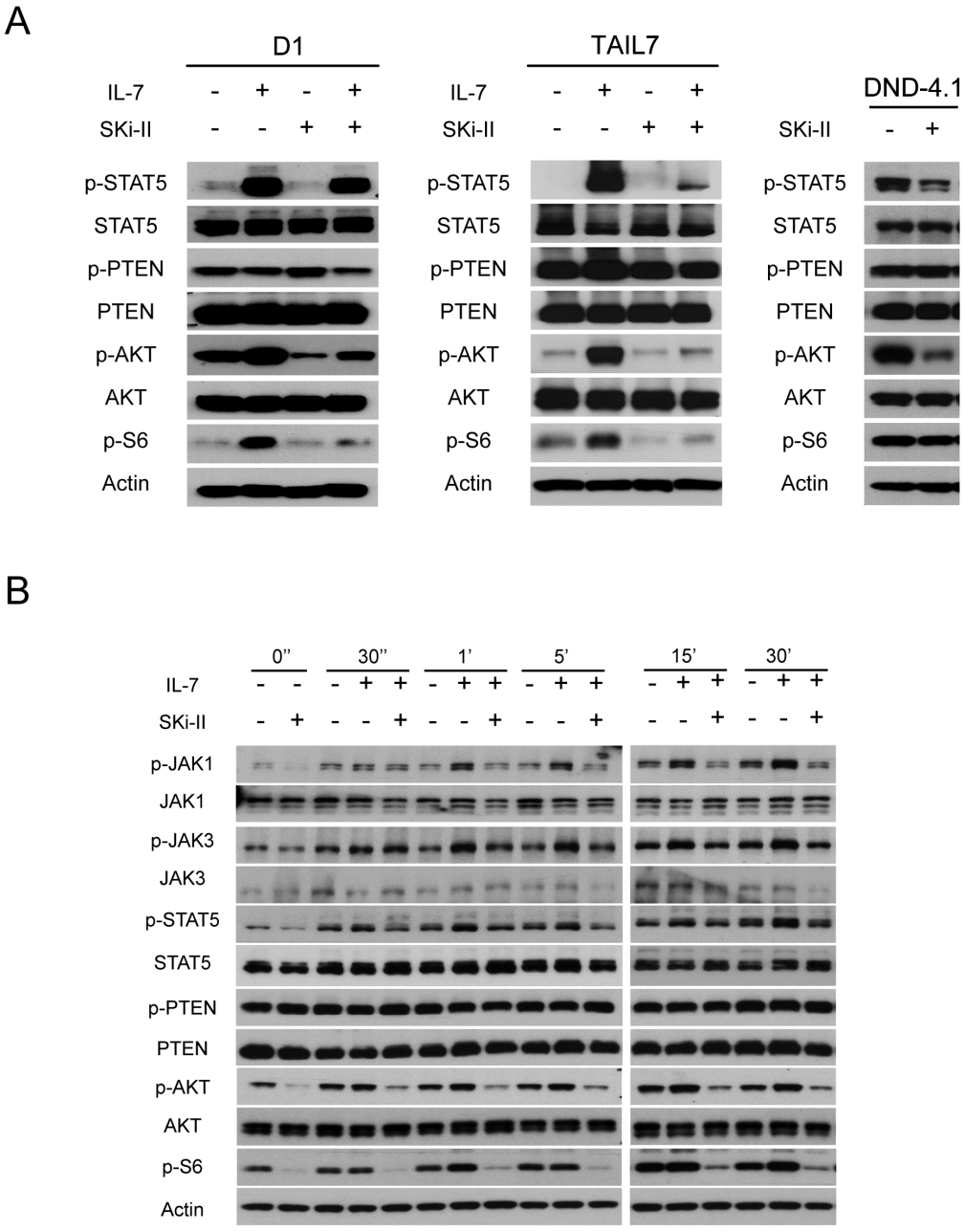

Figure 5: **SKI-II decreases STAT5, AKT and S6 phosphorylation in IL-7 mediated signaling.** (A) D1 cells were starved for 2h and total protein extracts were collected after 2h incubation with 10μM of SPHK1 specific inhibitor and 15 min in the presence of 50 ng/ml of IL-7. TAIL7 cells were starved for 24h and total protein extracts were collected after 2h incubation with 10μM of SPHK1 specific inhibitor and 15 min in the presence of 20ng/ml of IL-7. DND-4.1 total protein extracts were collected after 2h incubation in the presence/absence of 10μM of SPHK1 specific inhibitor. STAT5, AKT, PTEN and S6 total and phosphorylated and Actin were analysed (B) D1 cells were starved for 2h and total protein extracts were collected after 2h incubation with SPHK inhibitor or vehicle and in the presence or absence of IL-7 for 30 seconds; 1, 5, 15 and 30 minutes. JAK3, JAK1, STAT5, AKT, PTEN total and phosphorylated and Actin proteins were analysed

4.3. Sphingosine Kinase inhibition reduces cell survival via enhancing caspase-dependent apoptosis

Next, we sought to evaluate the functional impact of our molecular observations. We found that SPHK inhibition decreases cell survival in normal murine thymocytes (p = 0.0003) and human T-ALL cells (p < 0.0001, Figure 6). This effect was dose- (Figure 6A) and time-dependent (p < 0.0001, Figure 6B).

To characterize the cell death mechanism triggered by SPHK inhibition, we performed annexin V/ 7AAD staining, in order to evaluate whether apoptosis was involved. The different cell populations could be identified as follows: viable (7AAD and annexin V double-negative), early apoptotic (annexin V positive, 7AAD negative), and late apoptotic/necrotic (7AAD and annexin V double-positive) cells. Inhibition of SPHK led to an increase in the frequency of early and late apoptotic cells (Figure 7A), both of which contribute to increase the percentages of apoptotic cells in D1 (p = 0.0265), TAIL7 (p < 0.0001) and DND-4.1 (p = 0.0005, Figure 7B). The process of apoptosis or programmed cell death (PCD) can also be defined by caspase-3 and PARP cleavage. In accordance with the annexin V/ 7AAD flow cytometry data, western blot analysis of DND-4.1 cells after 24h of SKI-II treatment showed increased cleavage of both caspase 3 and PARP (Figure 7C).

Mitochondrial membrane potential was described as an important parameter of mitochondrial function used as a very early indicator of apoptosis. The collapse of the mitochondrial transmembrane potential occurs at the same time of the opening of mitochondrial permeability transition pores, promoting cytochrome c release, which enhances other downstream events in the apoptotic cascade. As expected, SKI-II decreased mitochondrial membrane potential in D1 (p = 0.0427), TAIL7 (p = 0.0449) and DND-4.1 (p = 0.0002) cells (Figure 8).

Taken together, these results strongly indicate that SPHK activity is required for IL-7 mediated survival of T-ALL cells and murine thymocytes.

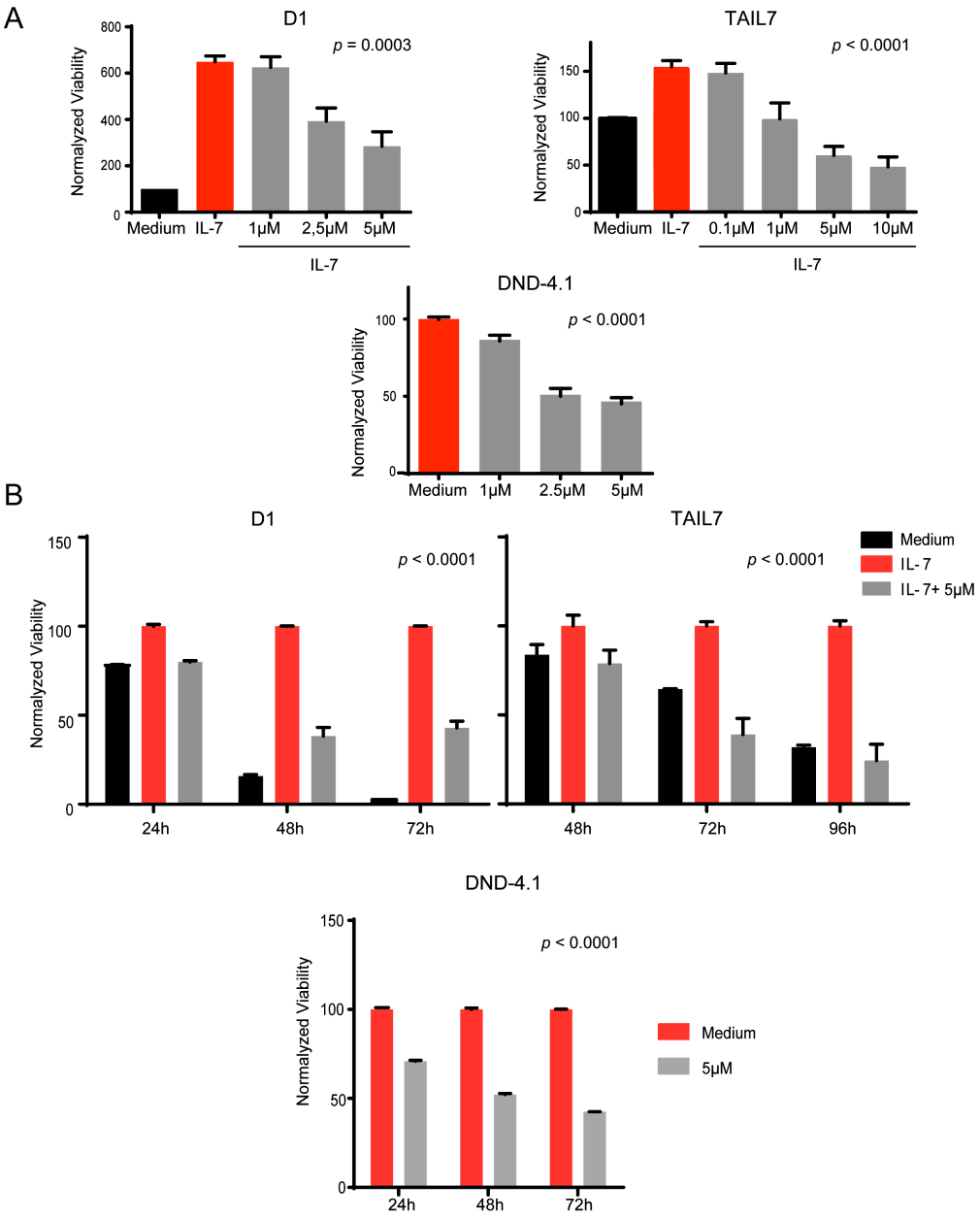

Figure 6: **Sphingosine Kinase inhibition reduces cell survival in normal and T-ALL cells.** (A) D1 and DND-4.1 cell viability was evaluated in the presence or absence of different concentrations (1, 2.5, 5 μM) of SKI-II. TAIL7 cell viability was evaluated in the presence or absence of different concentrations (0.1, 1, 5, 10 μM) of SKI-II. D1 and TAIL7 cells were cultured with 50 and 20ng/ml of IL-7, respectively. Data shown were obtained between 48h (D1 and DND-4.1) and 72h (TAIL7). Viability was obtained by flow cytometry with FSCxSSC analysis and normalized to medium conditions. Statistical analysis was performed using 2-Way ANOVA from duplicates of two experiments, $p = 0.0003$ and $p < 0.0001$. (B) D1, TAIL7 and DND-4.1 cell viability was evaluated in the presence or absence of different concentrations (5μM) of SKI-II. D1 and TAIL7 cells were cultured with 50 and 20ng/ml of IL-7, respectively. Viability was obtained by flow cytometry with FSCxSSC analysis and normalized to IL-7 condition in D1 and TAIL7 cell lines, and to medium condition in DND-4.1. Statistical analysis was performed using 2-Way ANOVA from duplicates of two experiments, $p < 0.0001$.

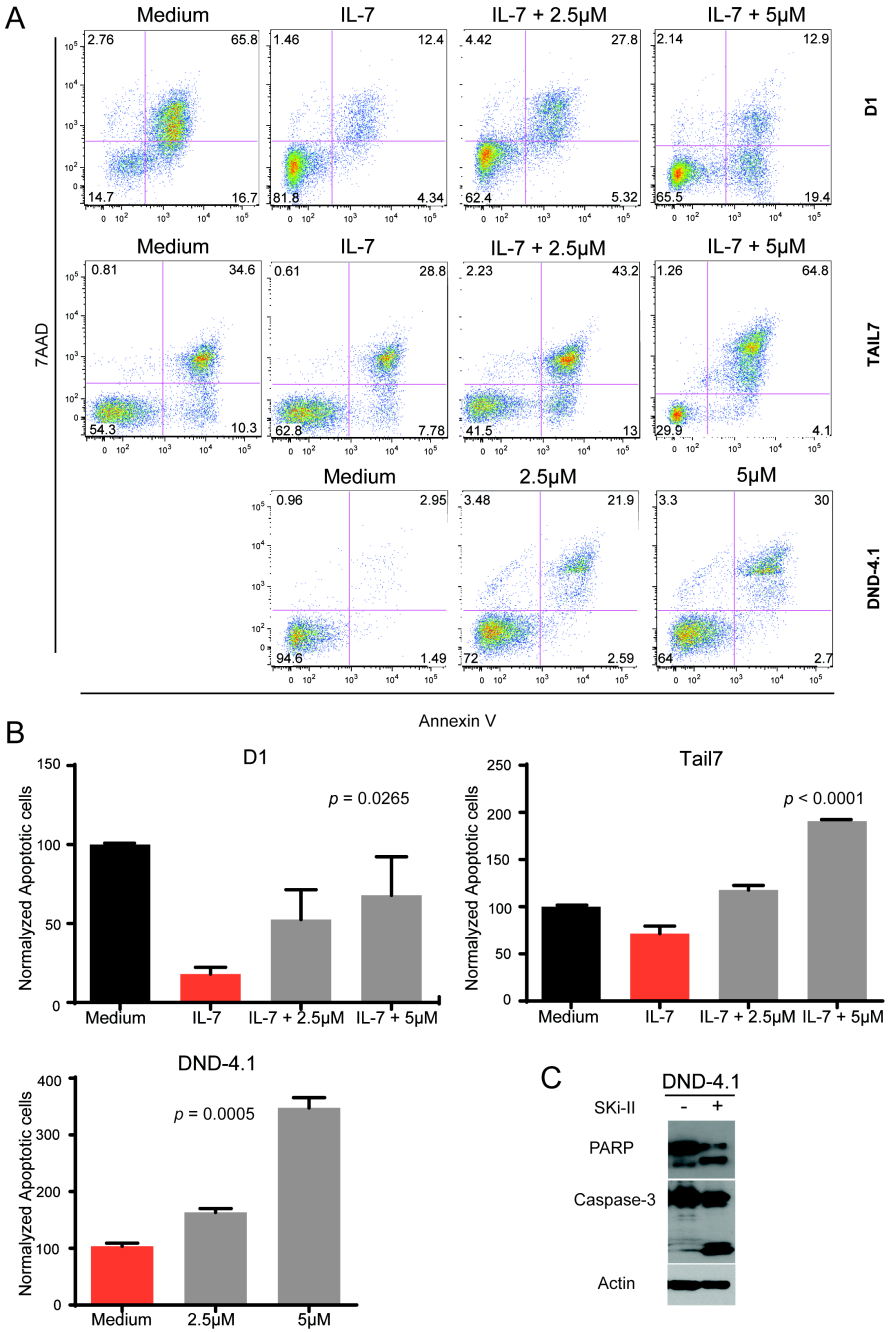

Figure 7: Sphingosine Kinase inhibition reduces cell survival by enhancing caspase dependent cell apoptosis. (A, B) Cells were cultured in the presence or absence of SKI-II (2.5 and 5μM). D1 and TAIL7 cells were cultured with 50 and 20ng/ml of IL-7, respectively. AnnexinV/7AAD staining assessed apoptotic and viable cells. Data shown was obtained between 48h (D1 and DND-4.1) and 72h (TAIL7). (B) Percentages of apoptotic cells were normalized to medium condition. Statistical analysis of D1 and TAIL7 cell lines were performed using 2-Way ANOVA from duplicates of two experiments, $p = 0.00265$ and $p < 0.0001$, respectively. Statistically significant differences of DND-4.1 were obtained through 1-Way ANOVA from duplicates of two experiments, $p = 0.0005$ is shown. (C) DND-4.1 total protein extracts were collected after 24h incubation in the presence/absence of 10μM of SPHK1 specific inhibitor. PARP and caspase-3 cleavage was analysed.

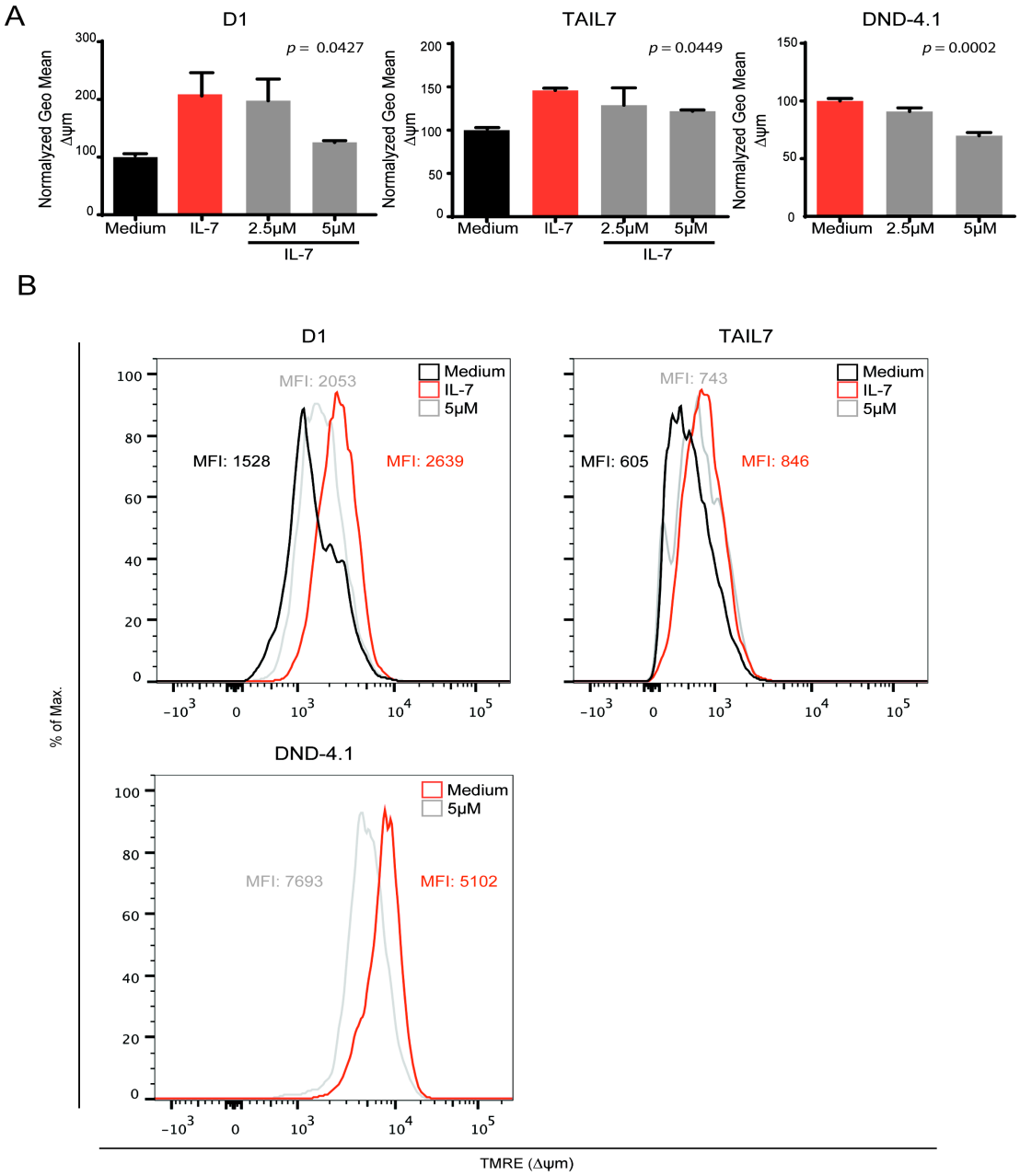

Figure 8: Sphingosine Kinase inhibition reduces Mitochondrial Membrane Potential. (A, B) Cells were cultured in the presence or absence of SKI-II (2.5 and 5μM). D1 and TAIL7 cells were cultured with 50 and 20ng/ml of IL-7, respectively. Mitochondrial Membrane Potential was assessed by TMRE staining. Data shown were obtained between 48h (D1 and DND-4.1) and 72h (TAIL7). (A) Geometric Mean of Mitochondrial Membrane Potential cells was normalized to medium condition. Statistically significant differences were obtained through 1-Way ANOVA from duplicates of two experiments, $p < 0.05$ is shown. (B) Representative histograms of Mitochondrial Membrane Potential measurement are shown. Mean Fluorescence Intensity (MFI) from each condition is shown.

4.4. Sphingosine Kinase inhibition prevents cell cycle progression and reduces cell proliferation

Since IL-7 has been shown to promote not only viability but also cell cycle progression of T-ALL cells, the effects of SKI-II on cell cycle and proliferation after IL-7 stimulation were also measured. SKI-II treatment decreased cell proliferation of IL-7-cultered TAIL7 cells (p = 0.0002) and DND-4.1 cells (p = 0.0189, Figure 9A). The same results were obtained for D1 cell line (data not shown). Regarding the effects on cell cycle, SKI-II led to a cell cycle arrest in G0/G1 in TAIL7 and DND-4.1 cell lines (Figure 9B). Similar results were obtained for D1 cell line (data not shown).

These results indicate that SPHK activity is essential for IL-7-mediated cell cycle progression and proliferation.

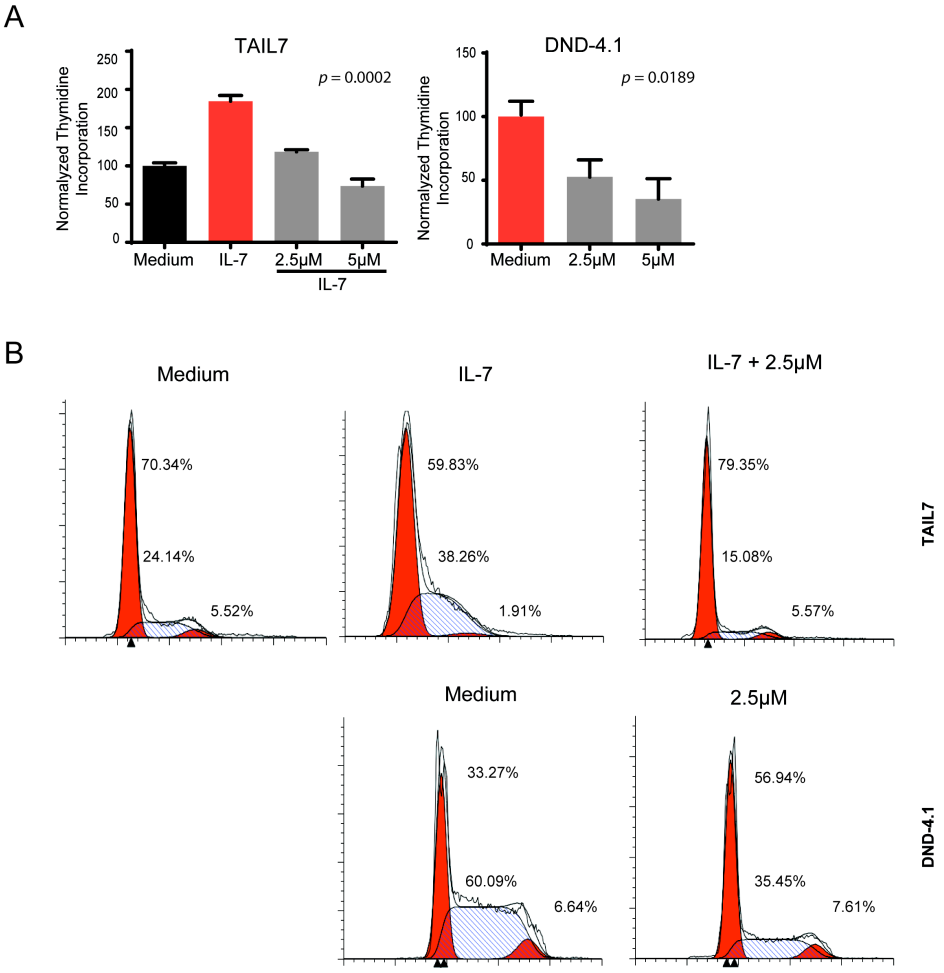

Figure 9: Sphingosine Kinase inhibition supresses cell proliferation and induces cell cycle blockage in T-ALL. (A, B) Cells were cultured in the presence or absence of SKI-II (2.5 and 5μM). TAIL7 cells were cultured with 20ng/ml of IL-7. (A) Data shown were obtained between 48h (DND-4.1) and 72h (TAIL7). Thymidine incorporation values were normalized to medium condition. Statistical analysis of TAIL7 and DND-4.1 cell lines were performed using 1-Way ANOVA from duplicates of two experiments, p = 0.0002 and p = 0.0189, respectively is shown. (B) Data shown were obtained between 24h (DND-4.1) and 48h (TAIL7). Cell cycle phase percentages were evaluated by flow cytometry analysis of PI staining in ethanol-fixed and permeabilized cells.

4.5. Sphingosine Kinase inhibition decreases cell size and transferrin receptor expression

IL-7 was shown to promote cell size increase (cell growth), which associates with increased metabolism and expression of 'activation' markers such as the transferring receptor CD71. Inhibition of SPHK impaired IL-7-mediated TAIL7 cell growth cells (p = 0.0109) and promoted the atrophy of DND-4.1 cells (p = 0.0016, Figure 10A). Surprisingly, in contrast to T-ALL cells, SKI-II treatment increased the cell size of D1

murine thymocytes (p = 0.0041, Figure 10A). In accordance, we found that SPHK inhibition was negatively affected CD71 expression in both TAIL7 (p = 0.0324) and DND-4.1 cells (p < 0.0001, Figure 10B). In agreement with the cell size response, CD71 was also more expressed after SKI-II treatment in D1 cells (p = 0.0025, Figure 10B).

These results suggest that SPHK inhibition decreases cell size and transferrin receptor expression selectively in T-ALL cells.

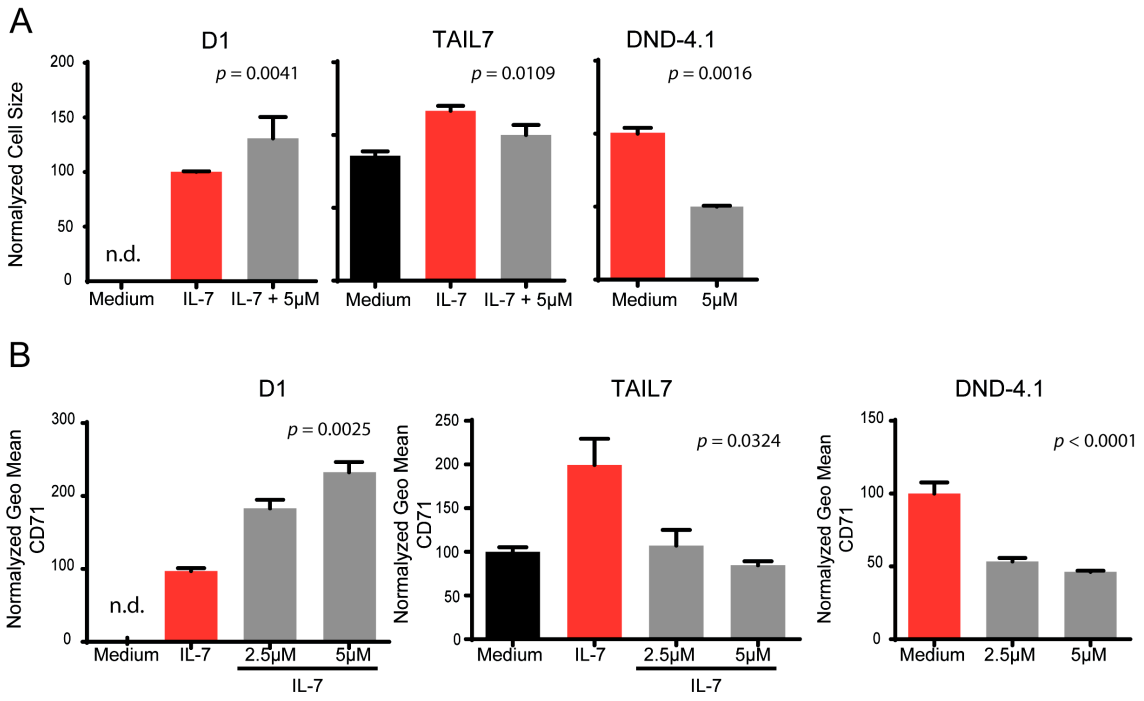

Figure 10: Sphingosine Kinase inhibition decreases cell size and transferrin receptor expression. (A, B) Cells were cultured in the presence or absence of SKI-II (2.5 and 5μM). TAIL7 cells were cultured with 20ng/ml of IL-7. Data shown were obtained between 48h (D1 and DND-4.1) and 72h (TAIL7). (A) Cell size was obtained by flow cytometry with FSCxSSC analysis. TAIL7 and DND-4.1 were normalized to medium conditions. D1 values were normalized to IL-7 condition, due to the lower % of viable cells in medium condition, we were not able to measure cell size (n.d. - no data). Statistical analysis was performed using 1-Way ANOVA from a representative experiment, p = 0.0041, p = 0.0109 and p = 0.0016, respectively. (B) Transferrin receptor (CD71) expression was evaluated by specific antibody staining and analyzed by flow cytometry. TAIL7 and DND-4.1 were normalized to medium condition. D1 values were normalized to IL-7 condition, due to the lower % of viable cells in medium condition, we were not able to measure CD71 (n.d. - no data). Statistical analysis was performed using 1-Way ANOVA from a representative experiment, p = 0.0025, p = 0.0324 and p < 0.0001, respectively.

DISCUSSION

The critical role of SPHKs in the development of a wide range of tumors has been exhaustively demonstrated, including in breast, lung, ovary, stomach, uterus, and kidney cancers, and both chronic and acute myeloid leukemias (99, 100). It is well-known that SPHK1 expression or/and activity are increased in cancer cells, being related with cell survival and proliferation (79). In contrast, SPHK2 function is still controversial, apparently displaying two contrasting functions under different conditions. In mouse embryonic kidney cells (HEK293) or mouse embryonic fibroblasts, SPHK2 silencing with small interfering RNA (siRNA) prevented the induction of apoptosis (121). In opposition, in human breast cancer cells, SPHK2 knockdown inhibited the growth of xenografted tumours (113). SPHKs subcellular localization has been pointed out as the decisive factor, nonetheless additional experiments are required (76).

Recent studies have established the importance of SPHKs in hematological malignancies. In Multiple Myeloma (MM), IL-6 stimulation leads to increased SPHK1 expression and activity, which upregulates myeloid cell leukaemia (Mcl-1), increasing cell proliferation and survival (106). Recently, it has been demonstrated that SPHK2 activity, but not its expression, was higher in ALL samples than normal B-cell progenitors (116). In this work, we show that SPHK1, but not SPHK2, may have an important role in T-ALL pathology since it is significantly overexpressed in malignant samples. In agreement with this notion, it has been shown that SPHK inhibition promotes apoptosis of human T-ALL Jurkat cells (110), suggesting that SPHK overexpression in T-ALL cells is of functional relevance.

SPHK has been indicated as a cytokine-activated kinase. As stated in the previous paragraph, IL-6 stimulates SPHK1 activity and leads to a decrease in both sphingosine and ceramide levels, which leads to suppression of cell apoptosis in MM (106). Furthermore, ERK1/2 and PI3K/mTOR are the main players responsible for SPHK1 phosphorylation and activation (106). Constitutive activation of IL-7 pathway, which drives the activation of JAK/STAT and both of these pathways (63), has been associated with leukemogenesis in T-ALL. In that sense, IL-7 signaling emerges as an obvious candidate for SPHK activation, and in fact we demonstrated that IL-7 stimulation leads to increased activity of both SPHK1 and SPHK2. It remains, however, to be understood what is the functional role of each of the two kinases in the context of IL-7-mediated signaling.

Previously, it has been reported that SPHK translocation from the cytosol to the plasma membrane is absolutely required for SPHK activation. These data suggest that plasma membrane localization is essential not only for SPHK activation, thus placing SPHK in close proximity to its substrate, sphingosine, but also to facilitate its product, S1P, acting on intracellular signaling targets or moving to the extracellular compartment and stimulating its G protein-coupled receptors (88). Here, we demonstrated for the first time that SPHK has a critical role in IL-7 and IL-7R-mediated signaling, and is absolutely required for the activation of downstream elements in IL-7 signaling pathways in normal T-cell precursors and T-ALL cells. Before, it was proposed that IL-7Rα association with clathrin and subsequent internalization is necessary for optimal IL-7-mediated signal transduction (122). The time-course experiments we now conducted showed that SPHK inhibition has an early effect on IL-7 signaling, which could indicate that SPHK is placed very upstream, at close proximity to the JAKs and PI3K - possibly even physically interacting with them or with the IL-7R. Alternatively, SPHK inhibition may promote changes in lipid membrane composition and by this way decrease IL-7 internalization, compromising IL-7-mediated signaling. Further experiments are required to clarify if and what are the molecular mechanisms by which SPHK may affect IL-7R internalization. On the other hand, SKI-II inhibits the conversion of sphingosine and ceramide into S1P, promoting intracellular accumulation of ceramide and sphingosine. This elevation of ceramide activates serine/threonine protein phosphatases PP1 and PP2A that dephosphorylate Akt (120). Akt deactivation is mediated not only by IL-7 signaling shutdown but also by increased ceramide, which could explain the effect of SKI-II in the regulation of PI3K/AKT pathway activation. This, however, would not justify the impact on JAK/STAT pathway.

Importantly, in agreement with its effects on key survival pathways, we further demonstrate that in T-ALL and normal thymocytes, SPHK is absolutely required for IL-7-mediated pro-survival effects. Previously it was demonstrated that IL-7 induces the phosphorylation of Akt, which interacts with its downstream targets GSK-3, FOXO1, and FOXO3a in a PI3K-dependent manner, leading to their inactivation and nuclear export, which promote cell viability. In addition, GSK-3 can also phosphorylate and inhibit NF-ATc, a transcription factor involved in proliferation and Bcl-2 gene transcription (63). It was also well shown that sphingosine rheostat is responsible for apoptosis regulation. Death-inducing signals reach the mitochondria, promoting cytocrome c (cyt c), adaptor of protein Apaf-1, and precursor of caspase-9, Smac diablo and dATP release. Pro-caspase 9 is cleaved and activates other caspases, such

as caspase-3, -6 or -7, which lead to apoptosis. Some reports indicate that increased levels of ceramide are responsible for mediating Fas- and TNF-α-induced apoptosis and precede previously described mitochondrial events in different stress conditions. S1P has an opposite effect, inhibiting cyt c and Smac/Diablo translocation to the cytoplasm, thereby preventing apoptosis. These findings suggest that a combination of both conditions, decreased S1P formation and increased ceramide levels, will promote cell apoptosis, which is achieved through SPHK inhibition (110). Our data is consistent with these reports, since SKI-II induced mitochondrial transmembrane potential loss, caspase-3 and PARP cleavage, and consequent apoptosis of malignant as well as normal T-cell precursors. Of note, mitochondrial transmembrane potential loss has been observed during ceramide-induced effector phase of apoptosis.

In non-transformed NIH 3T3 fibroblasts, sphingosine kinase transfection significantly increases the proliferative rate of fibroblasts (123). Our data demonstrated that SPHK inhibition decreases the proportion of cells in S-phase and blocks the G1/S transition in IL-7 stimulated normal and T-ALL cells. This could be explained possibly by the negative effect of SKI-II on proliferative signals, such as Cyclin-dependent Kinase (Cdc2 and Cdk2 kinases) expression, which can also be regulated by IL-7-mediated signaling (22). GSK-3β, which is inactivated by phosphorylation by Akt, is described as responsible for cyclin D1 regulation, contributing to cell cycle arrest. Since SPHK inhibition represses the activation of IL-7-mediated pathways, cell cycle related molecules are also expected to be repressed, explaining potentially the effect seen in T-ALL cells and normal T-cells after SPHK inhibition.

There are evidences that suggest a relation between increased cell size, cell metabolism and oncogenesis. TFR1 (CD71) is upregulated in order to supply increased cell metabolism requirements. Previously it was shown that IL-7 contributes to T-ALL cell growth and metabolic activation, increasing cell size and surface expression of CD71. Those events are dependent on PI3K/Akt activation (63). Recently, Pham et al. showed that SPHK1 expression leads to increase of CD71 surface levels and transferrin uptake in a manner that appears to be dependent also on c-Myc (124). We showed that SKI-II decreases CD71 expression and cell size in T-ALL cells. It remains to be determined if this effect is dependent on SPHK1-mediated activation of PI3K pathway. Surprisingly, SPHK inhibition upregulated CD71 surface expression and cell size in normal thymocytes, represented by the mouse D1 cell line. It is possible that normal T-cell precursors differ from T-ALL cells in IL-7-mediated, SPHK-dependent regulation of metabolism - an observation that may be of therapeutic importance. It

should be noted, nonetheless, that experiments involving primary human thymocytes are required to confirm the data obtained so far with D1 cells.

CONCLUSION

IL-7 and its receptor are crucially involved in T-cell leukemia pathophysiology. The current study provides evidence that IL-7/IL-7R-mediated signaling upregulates SPHK1 kinase activity and absolutely requires SPHK for full activation of downstream effectors. The activation of IL-7 pathway is not only related with increased viability, but also involved in the regulation of cell size and proliferation. Altogether, our results indicate that SPHK plays an important role in IL-7-mediated signaling and its downstream effects on T-ALL cells. Together with our evidence that SPHK1 is overexpressed in T-ALL cells, these findings could constitute a solid argument for the further deepening of the understanding of the role of SPHK in T-ALL, which could help define whether SPHK is a valid molecular target for therapeutic intervention in T-ALL. To complement our *in vitro* studies, analysis of inhibition of SPHK in primary T-ALL samples should be performed and *in vivo* efficacy of inhibitors of SPHK for the treatment of T-ALL should be tested.

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