Activation of PI3K Is Indispensable for Interleukin 7–mediated Viability, Proliferation, Glucose Use, and Growth of T Cell Acute Lymphoblastic Leukemia Cells

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Abstract

Interleukin (IL)-7 is essential for normal T cell development. Previously, we have shown that IL-7 increases viability and proliferation of T cell acute lymphoblastic leukemia (T-ALL) cells by up-regulating Bcl-2 and down-regulating the cyclin-dependent kinase inhibitor $p27^{kip1}$. Here, we examined the signaling pathways via which IL-7 mediates these effects. We investigated mitogen-activated protein kinase (MEK)–extracellular signal-regulated kinase (Erk) and phosphatidylinositol-3-kinase (PI3K)–Akt (protein kinase B) pathways, which have active roles in T cell expansion and have been implicated in tumorigenesis. IL-7 induced activation of the MEK–Erk pathway in T-ALL cells; however, inhibition of the MEK–Erk pathway by the use of the cell-permeable inhibitor PD98059, did not affect IL-7–mediated viability or cell cycle progression of leukemic cells. IL-7 induced PI3K-dependent phosphorylation of Akt and its downstream targets GSK-3, FOXO1, and FOXO3a. PI3K activation was mandatory for IL-7–mediated Bcl-2 up-regulation, $p27^{kip1}$ down-regulation, Rb hyperphosphorylation, and consequent viability and cell cycle progression of T-ALL cells. PI3K signaling was also required for cell size increase, up-regulation of CD71, expression of the glucose transporter Glut1, uptake of glucose, and maintenance of mitochondrial integrity. Our results implicate PI3K as a major effector of IL-7–induced viability, metabolic activation, growth and proliferation of T-ALL cells, and suggest that PI3K and its downstream effectors may represent molecular targets for therapeutic intervention in T-ALL.

Key words: T cell acute lymphoblastic leukemia • IL-7 • PI3K–Akt • MEK–Erk • Glut1

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 up-regulating Bcl-2 expression (1). Similar to normal immature thymocytes, leukemic blasts from T cell acute lymphoblastic leukemia (T-ALL), patients can express functional IL-7Rs (2). Moreover, different studies demonstrated that IL-7 can induce proliferation and prevent spontaneous apoptosis of T-ALL cells in vitro (2, 3). Importantly, IL-7 is present in the microenvironments where the leukemia arises because it is produced by thymic epithelial and bone marrow stromal cells (4, 5), and significantly contributes to the increased survival of T-ALL cells cocultured with thymic epithelial cells (6). These data suggest that IL-7 may play an important role in the biology of T-ALL. We have shown previously that IL-7 down-regulates the expression of the cyclin-dependent kinase (cdk) inhibitor $p27^{kip1}$, leading not only to cell cycle progression but also to up-regulation of Bcl-2 protein expression and viability of T-ALL cells (7). Furthermore, IL-7 can support the long-term expansion of primary T-ALL cells, as shown by the establishment an IL-7–

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Abbreviations used in this paper: cdk, cyclin-dependent kinase; Erk, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase; MIF, mean intensity of fluorescence; PI3K, phosphatidylinositol-3-kinase; T-ALL, T cell acute lymphoblastic leukemia.

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dependent cell line (TAIL7) that maintains the essential features of IL-7–responsive primary T-ALL blasts (8).

Mitogen-activated protein kinase (MEK)–extracellular signal-regulated kinase (Erk) (MEK–Erk) signals have been shown to promote viability (9) and induce cell cycle progression by regulating the expression of c-Myc, cyclin D1, $p27^{kip1}$, and $p21^{cip1}$ (10). Phosphatidylinositol-3-kinase (PI3K) and Akt/protein kinase B (hereafter referred to as Akt) have also been associated with prevention of apoptosis and cell cycle progression (11–13). These effects are implicated in PI3K–Akt-mediated tumorigenesis (12, 14–16). Strikingly, Jurkat and other T cell leukemia cell lines lack PTEN and/or SHIP, and consequently have high PI3K and Akt basal activities (17). Both MEK–Erk and PI3K–Akt pathways are involved in T cell survival, expansion, and differentiation (18–22). However, IL-7 appears to activate PI3K–Akt but not MEK–Erk in normal T cells (18, 23).

Our present studies demonstrate that IL-7 activated the MEK–Erk pathway in T-ALL cells, contrary to what occurs in normal T cells (18, 23). However, MEK–Erk did not appear to be essential for IL-7–mediated viability and proliferation of T-ALL cells. IL-7 also triggered PI3K-dependent phosphorylation of Akt, GSK-3, FOXO1, and FOXO3a. Inhibition of PI3K prevented up-regulation of Bcl-2, down-regulation of $p27^{kip1}$, and hyperphosphorylation of Rb, and abrogated IL-7–mediated survival and proliferation of T-ALL cells. IL-7 induced expression of the glucose transporter Glut1 in a PI3K-dependent fashion, and this event correlated with glucose use, mitochondrial integrity, increase of cell size, and up-regulation of CD69 and CD71. These results demonstrate that PI3K downstream signals are fundamental for IL-7–mediated survival, activation, proliferation, and growth of T-ALL cells, and may regulate clonal expansion of T cell acute leukemia.

Materials and Methods

Primary T-ALL Samples and the 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, washed twice in RPMI 1640 supplemented with 10% (vol/vol) FBS and 2 mM L-glutamine (hereafter referred to as RPMI 10 medium), subjected to immunophenotypic analysis by flow cytometry as described previously (7), and classified according to their maturation stage using the criteria defined by the European Group for Immunological Characterization of Leukemias (see Table I; reference 24). The TAIL7 cell line was established from the peripheral blood of a pediatric T-ALL patient. It is IL-7 dependent and has been described previously (8).

In Vitro Culture. Primary T-ALL or TAIL7 cells isolated by density centrifugation over Ficoll-Hypaque were cultured in 24-well plates at 2×10^6 cells/ml at 37°C with 5% CO_2 in the following: RPMI 10 (control medium), 10 ng/ml IL-7 (Endogen), IL-7 plus 10 μM MEK-specific inhibitor PD98059 (Calbiochem), or IL-7 plus 10 μM PI3K-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 d or in RPMI without FBS for 1–4 d, with similar results.

Proliferation Assays. Cells were cultured in triplicates in flat-bottom 96-well plates at 2×10^6 cells/ml at 37°C with 5% CO_2 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 h before harvest. DNA synthesis, as measured by [^{3}H]thymidine incorporation, was assessed using a liquid scintillation counter. Average and standard deviation of triplicates were calculated.

Assessment of Cell Viability, Size, and Activation. Quantitative determination of viability of the malignant cells was performed using an annexin V–based apoptosis detection kit (R&D Systems), as described previously (7). Cell size was assessed by analysis of SSC versus FSC flow cytometry plots gated on the live cell population. Surface expression of activation markers CD71 and CD69 was measured by flow cytometry using FITC-conjugated anti-CD71 (DakoCytomation) and PE-conjugated anti-CD69 (Beckman Coulter) antibodies and appropriately matched isotype controls. Samples were analyzed using a FACSCalibur flow cytometer and CELLQuest software (Becton Dickinson). Results were expressed as the percentage of positive cells as compared with 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. In brief, 5×10^5 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. Analysis of flow cytometry cell cycle histograms was performed using ModFit LT software (Verity).

Short-Term Stimulation with IL-7. For the initial experiments, IL-7–deprived TAIL7 cells were washed twice with PBS and incubated for the indicated periods at 37°C with prewarmed PBS alone or with the indicated concentrations of IL-7. IL-7–deprived TAIL7 cells were then incubated for 15 min at 37°C with PBS alone or with 50 ng/ml IL-7. In defined experiments, the cells were pretreated in PBS with 10 μM LY294002, 10 μM PD98059, or the corresponding volume of vehicle (DMSO) for 2 h before 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. After the indicated conditions and time intervals of culture, cell lysates were prepared and equal amounts of protein were analyzed by 10% SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with the following mAbs or antiserum: $p27^{kip1}$ (BD Transduction Laboratories), actin, STAT5, and Glut1 (Santa Cruz Biotechnology, Inc.), ZAP-70 and phospho-STAT5A/B (Y694/Y699; Upstate Biotechnology), and phospho-Akt (S473), phospho-GSK-3β (S9), phospho-FKHR(FOXO1) (T24)/phospho-FKHRL1(FOXO3a) (T32), phospho-MEK1/2 (S217/S221), phospho-Erk1/2 (T202/Y204), Akt, and Erk1/2 (Cell Signaling Technology). To examine the phosphorylation status of Rb, proteins were analyzed by 6% SDS-PAGE, transferred onto nitrocellulose membrane, and blotted with Rb-specific mAbs (BD Biosciences). After immunoblot-

ting 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), as indicated by the host origin of the primary antibody and developed by chemiluminescence (Amersham Biosciences).

Akt in vitro kinase reactions were performed using a nonradioactive Akt kinase assay kit purchased from Cell Signaling Technology according to the manufacturer's instructions. In brief, cell lysates with equal amounts of protein were immunoprecipitated using agarose hydrazide–conjugated Akt antibody, washed twice, and resuspended in kinase buffer supplemented with 200 μM 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 was 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).

Intracellular Staining. Bcl-2 protein expression was assessed by intracellular staining. Cells were fixed in 0.1% formaldehyde for 30 min at 4°C, washed in PBS, resuspended in 1× Perm/Wash Solution (BD Biosciences), and incubated with mouse monoclonal FITC-conjugated anti–Bcl-2 antibody (DakoCytomation). Irrelevant isotype-matched antibody was used as negative control. 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.

Assessment of Mitochondrial Membrane Potential ($_{\Delta}\psi_m$). Cells were harvested, stained in culture medium with TMRE (Sigma-Aldrich) to a final concentration of 100 nM, and incubated for 30 min at 37°C with 5% CO_2. CCCP (Sigma-Aldrich) was added to duplicate tubes to a final concentration of 50 μM to collapse $_{\Delta}\psi_m$ and therefore validate the assay and serve as a control for background levels of fluorescence. Cells were analyzed for TMRE intensity by flow cytometry.

Glucose Uptake Assay. After the indicated culture conditions, 10^6 TAIL7 cells were starved in PBS at room temperature for 30 min and incubated at 37°C for 10 min in PBS containing 5 μM 2-{^{14}C(U)}-deoxy-D-glucose (PerkinElmer). Cells were harvested on filtermats and counted for ^{14}C-glucose content. Average and standard deviation of triplicates were calculated.

Online Supplemental Material. In Fig. S1, primary T-ALL cells were cultured with 10 ng/ml IL-7, either alone or in the presence of 10 μM PD098059 or 10 μM LY294002. Viability and proliferation were assessed as described above. Fig. S1 is available at http://www.jem.org/cgi/content/full/jem.20040789/DC1.

Results

IL-7 Activates MEK–Erk and PI3K–Akt Pathways in T-ALL Cells. PI3K–Akt and MEK–Erk pathways are thought to play an active role in normal thymocyte and mature T cell expansion (18–20). For this reason we investigated whether these pathways are involved in IL-7–mediated proliferation and viability of T-ALL cells that we have previously reported. We used both primary T-ALL samples and TAIL7, a cell line that displays IL-7–mediated responses identical to primary leukemia cells (8) and provides a useful tool for experiments that require high cell numbers that cannot be obtained using primary leukemia cells. Cytokine-deprived TAIL7 cells were stimulated by 50 ng/ml

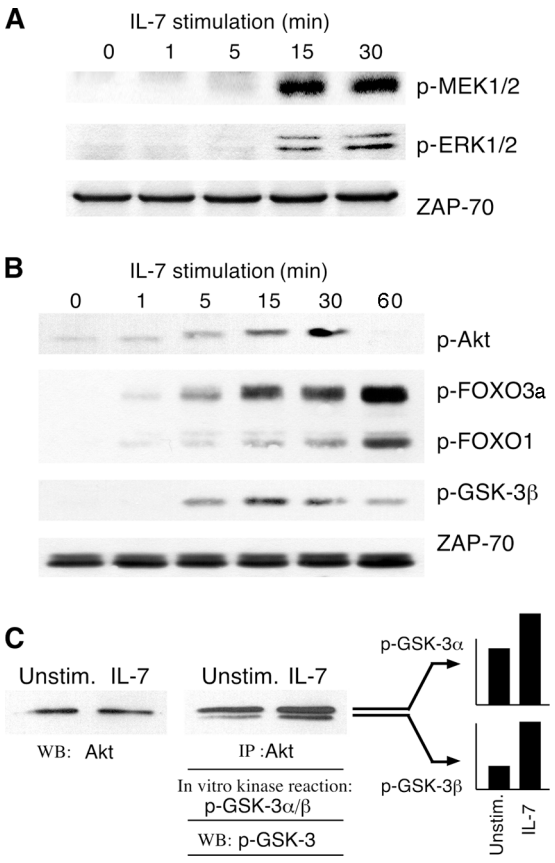

Figure 1. IL-7 activates MEK–Erk and PI3K–Akt pathways in T-ALL. IL-7–deprived TAIL7 cells were stimulated with IL-7 for the indicated periods (A and B). Cell lysates were resolved with 10% SDS-PAGE and immunoblotted with the indicated antibodies. Results representative of three independent experiments are shown. Levels of phosphorylated MEK1/2 and Erk1/2 were detected with antisera that selectively recognize the activated forms of the following kinases: Ser217/Ser221-phosphorylated MEK1/2 (P-MEK1/2) and Thr202/Tyr204-dual-phosphorylated Erk1/2 (P-Erk1/2). Levels of phosphorylated Akt and GSK-3 were analyzed with antibodies that specifically recognize Ser473-phosphorylated Akt (P-Akt) and Ser9-phosphorylated GSK-3β (P-GSK-3β), respectively. Levels of phosphorylated FOXO1/FOXO3a were detected with antiserum that reacts with Thr24-phosphorylated FOXO1 (P-AFOXO1) and Thr32-phosphorylated FOXO3a (P- FOXO3a). Akt and Erk1/2 protein levels were assessed with specific antibodies and remained unchanged (not depicted). Blots were reprobed with an anti–ZAP-70 antibody to confirm even protein loading. (C) IL-7 activates Akt and induces in vitro phosphorylation of GSK-3 by Akt. IL-7–deprived TAIL7 cells were stimulated with IL-7 for 15 min. 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 performed using crosstide-GSK-3α/β as exogenous substrate. Reactions were analyzed by 12% SDS-PAGE, transferred to nitrocellulose membrane, and GSK-3 phosphorylation was detected by immunoblotting with anti–phospho-GSK-3α/β (Ser21/Ser9) antibody. Even loading was confirmed 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 a 3.07-fold increase in GSK-3β Akt-mediated phosphorylation. Results are representative of two independent experiments.

IL-7 for increasing periods of time for up to 120 min, and protein phosphorylation was assessed by Western blot. Erk1/2 was phosphorylated and activated upon IL-7 stimulation of TAIL7 cells, as determined by immunoblotting with an antibody that exclusively detects Erk1/2 when catalytically activated by phosphorylation at both Thr202 and Tyr204. Phosphorylation of Erk1/2 was detectable after 5 min of stimulation, increased at 15 min, peaked by 30 min (Fig. 1 A), and was still detected by 2 h of stimulation (not depicted). A similar pattern of phosphorylation was observed for MEK1/2 (Fig. 1 A). When TAIL7 cells were stimulated with increasing doses of IL-7, phosphorylation of MEK and Erk was detected with 1 ng/ml and reached a plateau at 10 ng/ml (not depicted).

Next, we analyzed whether IL-7 activated the PI3K–Akt pathway in TAIL7 cells. Phosphorylation of Akt at Ser473, which is necessary for full activation of Akt kinase, occurred within 1 min, peaked at 30 min, and was undetectable after 1 h of IL-7 stimulation (Fig. 1 B). Members of the FOXO family of transcription factors are direct targets of Akt (25). In TAIL7 cells, IL-7 induced phosphorylation of FOXO1 (at Thr24) and FOXO3a (at Thr32) in a time-dependent manner (Fig. 1 B). FOXO1 phosphorylation at Ser256 did not appear to be regulated by IL-7 in these cells (not depicted). GSK-3, another known downstream target of Akt, was also phosphorylated by stimulation with IL-7 (Fig. 1 B). IL-7 mediated a dose-dependent phosphorylation of Akt and its downstream targets, which was detected at <1 ng/ml and reached a plateau at 10 ng/ml (not depicted). These results suggest that Akt becomes enzymatically active after stimulation with IL-7. To confirm that IL-7 can induce the enzymatic activity of Akt in TAIL7 cells, lysates from unstimulated or 15 min IL-7–stimulated cells were immunoprecipitated with an anti-Akt antibody and an in vitro kinase reaction was performed using GSK-3α/β as exogenous substrate. Phosphorylation of GSK-3α, and more prominently of GSK-3β, was up-regulated (1.6- and threefold, respectively) by stimulation with IL-7 (Fig. 1 C). These results confirm that IL-7 induced Akt phosphorylation, leading to its enzymatic activation and consequent phosphorylation of GSK-3.

Because we observed that phosphorylation of Akt, GSK-3, and FOXO family members was mediated by IL-7, we sought to confirm that these events were dependent upon PI3K activation. IL-7–deprived TAIL7 cells were pretreated with 10 μM of the cell-permeable PI3K-specific inhibitor LY294002 or MEK-specific inhibitor PD98059 before IL-7 stimulation. LY294002 specifically abrogated phosphorylation of Akt, without affecting phosphorylation of Erk1/2 or STAT5, whereas PD98059 specifically inhibited phosphorylation of Erk1/2 without affecting phosphorylation of Akt or STAT5 (Fig. 2 A). Furthermore, LY294002 but not PD98059 inhibited GSK-3, FOXO1, and FOXO3a phosphorylation (Fig. 2 B). These findings indicate that IL-7–induced phosphorylation of Akt and downstream targets in T-ALL cells are dependent on PI3K activity and can be specifically disrupted by LY294002.

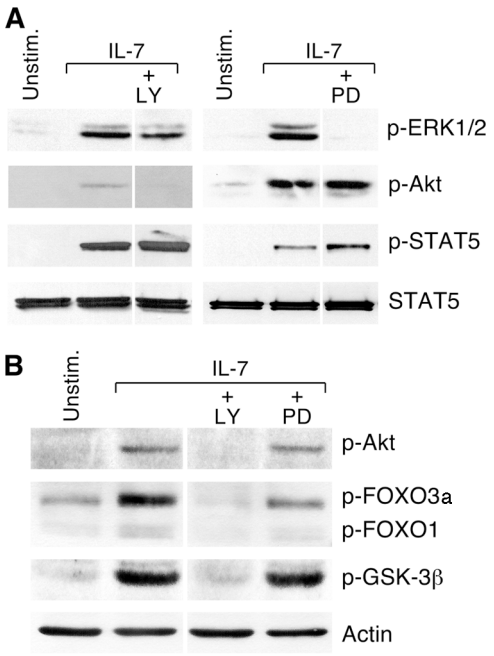

Figure 2. IL-7 induces PI3K-dependent phosphorylation of Akt, GSK-3, FOXO1, and FOXO3a, and MEK-dependent phosphorylation of Erk1/2 in T-ALL cells. IL-7–deprived TAIL7 cells were pretreated with 10 μM LY294002 (LY) or 10 μM PD098059 (PD) for 2 h, and then stimulated with IL-7 for 15 min. (A) Western blot analysis was performed with P-Erk1/2, P-Akt antibodies (see legend to Fig. 1), and an antibody specific for Tyr694/Tyr699-phosphorylated-STAT5A/B (P-STAT5) to confirm that LY294002 and PD98059 were specific inhibitors of the PI3K–Akt and MEK–Erk pathway, respectively. (B) GSK-3β, FOXO1, and FOXO3a phosphorylation is dependent on PI3K activity. Western blot analysis was performed with P-Akt, P-GSK3β, and P-FOXO1/FOXO3a antibodies. Anti-STAT5 (A) and actin (B) antibodies were used to confirm equal loading. Representative results from three independent experiments are shown.

PI3K But Not MEK 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 IL-7–mediated viability and proliferation of TAIL7 cells by using LY294002 and PD98059. Initial experiments determined that IL-7 stimulated a dose-dependent increase in viability and proliferation of TAIL7 cells, which was most evident between 72 and 96 h of culture with 10 ng/ml IL-7 (not depicted). Therefore, we used this concentration and time points in all subsequent experiments. 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 (Fig. 3 A). The same pattern was obtained when we examined cell proliferation by assessing the incorporation of [^{3}H]thymidine. As shown in Fig. 3 B, PD98059 did not affect proliferation of IL-7–cultured TAIL7 cells. In contrast, LY294002 completely abrogated IL-7–induced proliferation (Fig. 3 B). Next, we examined whether inhibition of PI3K prevented

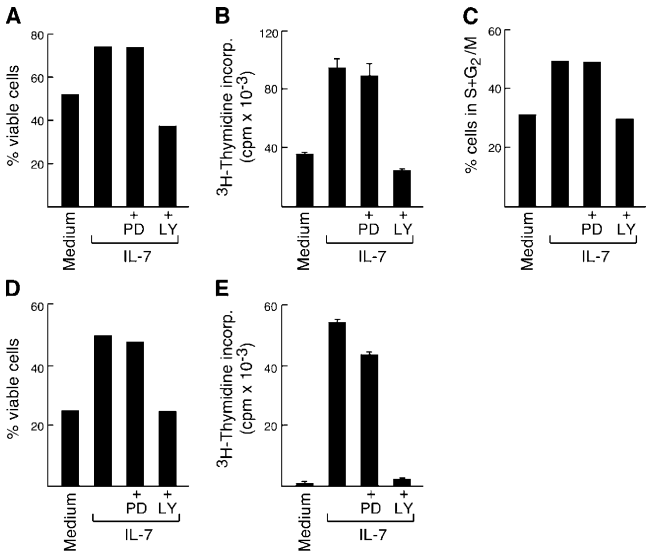

Figure 3. PI3K but not MEK is critical for IL-7–mediated viability and cell cycle progression of T-ALL cells. (A–C) TAIL7 cells were cultured for 96 h with 10 ng/ml IL-7, either alone or in the presence of 10 μM PD098059 (IL-7+PD) or 10 μM LY294002 (IL-7+LY). (A) TAIL7 cells were stained with annexin V–FITC plus propidium iodide and viability was determined by flow cytometry analysis. (B) Proliferation was determined by assessment of [^{3}H]thymidine incorporation. (C) Percentage of cells at S+G2/M phases of the cell cycle was determined by propidium iodide staining followed by flow cytometry analysis. (D and E) Primary T-ALL cells were obtained as described in Materials and Methods, and cultured under the indicated conditions. Viability at 96 h (D) and proliferation at 72 h (E) of culture were assessed as described for TAIL7 cells. Results are representative of three to six independent experiments with TAIL7 cell line and two independent experiments with all five primary T-ALL samples. Results from remaining primary T-ALL samples are shown in Fig. S1.

[^{3}H]thymidine incorporation only because it impaired cell viability, or whether it also blocked cell cycle progression. TAIL7 cells were fixed and stained with propidium iodide and analyzed for cell cycle progression by flow cytometry. As shown in Fig. 2 C, the PI3K inhibitor LY294002 abrogated cell cycle progression of IL-7–cultured cells, whereas the MEK inhibitor PD98059 had no effect. Thus, PI3K seems to have a dominant role in IL-7–mediated viability and cell cycle progression in TAIL7 leukemia cells.

To confirm the biological significance of these results obtained in TAIL7 cells, we examined whether PI3K played a similar critical role on IL-7–mediated effects in primary T-ALL cells. Leukemic cells were collected from the peripheral blood or bone marrow of pediatric T-ALL patients with high leukemia involvement and tested for in vitro responsiveness to IL-7 by assessing cellular proliferation. IL-7–responsive samples were immunophenotyped, classified according to their maturation stage (Table I), and used in subsequent experiments. The increase in viability (Fig. 3 D and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20040789/DC1) and proliferation (Fig. 3 E and Fig. S1) induced with IL-7 was completely abrogated by LY294002, whereas PD98059 had no significant impact on IL-7–mediated effects in primary leukemia cells. Our data demonstrate that PI3K is indispensable for both viability and proliferation mediated by IL-7 not only in the TAIL7 line, but also in primary T-ALL cells. In contrast, MEK–Erk pathway albeit activated by IL-7 does not appear to play a functionally significant role in mediating these effects of IL-7 in T-ALL.

IL-7–mediated Down-regulation of p27^{kip1}, Hyperphosphorylation of Rb, and Up-regulation of Bcl-2 in T-ALL Cells Is Dependent upon PI3K Activity. Cell cycle progression from G0/G1 to S phase is modulated by exogenous factors and is positively regulated by cyclin–cdk holoenzymes, which phosphorylate several intracellular substrates including Rb. Phosphorylation and inactivation of Rb result in release of E2F and transcription of genes that are required for progression through S phase (26). The enzymatic activity of cyclin–cdk complexes is negatively regulated by cdk inhibitors, including the cip/kip family member p27^{kip1} (27). We previously demonstrated that culture with IL-7 down-regulates 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-dependent events could link IL-7 to the cell cycle machinery. Culture of TAIL7 cells with IL-7 for 96 h resulted in down-regulation of p27^{kip1} (Fig. 4 A) and hyperphosphorylation of Rb (Fig. 4 B). LY294002 completely reversed these IL-7–mediated effects, up-regulating p27^{kip1} to the same levels as those

Table I. *Immunophenotype and Classification of T-ALL Patients*

	Immunophenotype													
T-ALL no.	CD1	CD2	CD3	CD4	CD5	CD7	CD8	CD10	CD14	CD19	CD33	CD34	CD56	Maturation stage
1	+	+	+	−	+	+	−	−	−	−	−	+	−	III
2	+	+	+	−	+	+	−	−	−	−	−	−	−	III
3	−	+	−	+	+	+	−	+	−	−	−	+	−	II
4	+	+	−	+	+	+	+	−	ND	ND	−	−	ND	III
5	−	+	−	+	+	+	−	+	−	−	−	+	−	II

T cell maturation stages of primary samples were defined as described previously (reference 24). Stage II, pre–T-ALL; stage III, cortical T-ALL.

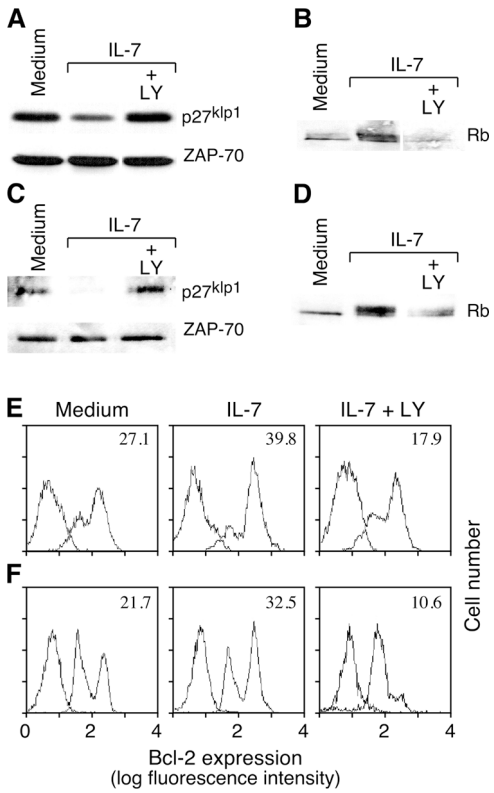

Figure 4. IL-7 mediates $p27^{kip1}$ down-regulation, Rb hyperphosphorylation, and Bcl-2 up-regulation via activation of PI3K in T-ALL cells. TAIL7 cells (A and B) or primary T-ALL cells (C and D) were cultured for 96 or 72 h, respectively, under the indicated conditions. (A and C) Cell lysates were 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 and D) Lysates from the same samples were analyzed by 6% SDS-PAGE and immunoblotted with an Rb-specific antibody. The hyperphosphorylated form of Rb corresponds to the band with the higher apparent molecular weight. Blasts from T-ALL number 3 were used in this experiment. (E) Bcl-2 protein levels at 96 h of culture were assessed by flow cytometry after intracellular staining of TAIL7 cells with FITC-conjugated anti–Bcl-2 antibody. (F) Expression of Bcl-2 in primary T-ALL cells was assessed at 72 h of culture. Results are representative of four different patient samples analyzed. Specific MIF, as described in Materials and Methods, is indicated in each histogram. Results were similar in six independent experiments.

presented by leukemic cells cultured in medium alone, and preventing Rb hyperphosphorylation. MEK inhibition using PD98059 did not affect $p27^{kip1}$ levels or Rb phosphorylation (not depicted). Similar results were observed for primary T-ALL cells (Fig. 4, C and D). These results indicate that PI3K activation is essential for IL-7 to induce $p27^{kip1}$ down-regulation and cell cycle progression in T-ALL cells.

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 (3). We subsequently demonstrated that Bcl-2 up-regulation was mandatory for IL-7–mediated promotion of viability. Moreover, we observed a causative link between $p27^{kip1}$ down-regulation and increase in Bcl-2 expression (7). Because the addition of LY294002 completely blocked IL-7–mediated viability of T-ALL cells, we examined whether IL-7 regulated Bcl-2 via activation of PI3K. TAIL7 cells were cultured with either medium alone, IL-7, or IL-7 plus LY294002. After 96 h, cells were analyzed for intracellular expression of Bcl-2 by flow cytometry. IL-7–mediated up-regulation of Bcl-2 was significantly impaired by LY294002 (Fig. 4 E). Primary T-ALL samples showed similar results (Fig. 4 F). Thus, up-regulation of Bcl-2 mediated by IL-7 in T-ALL cells requires activation of PI3K.

IL-7 Induces Increased Cell Size and Activation of T-ALL Cells in a PI3K-dependent Manner. T cell activation can be measured by increased cell size (cell growth) and by the surface expression of CD69 and CD71. After 72 h of culture, IL-7 strikingly up-regulated cell size of TAIL7 cells

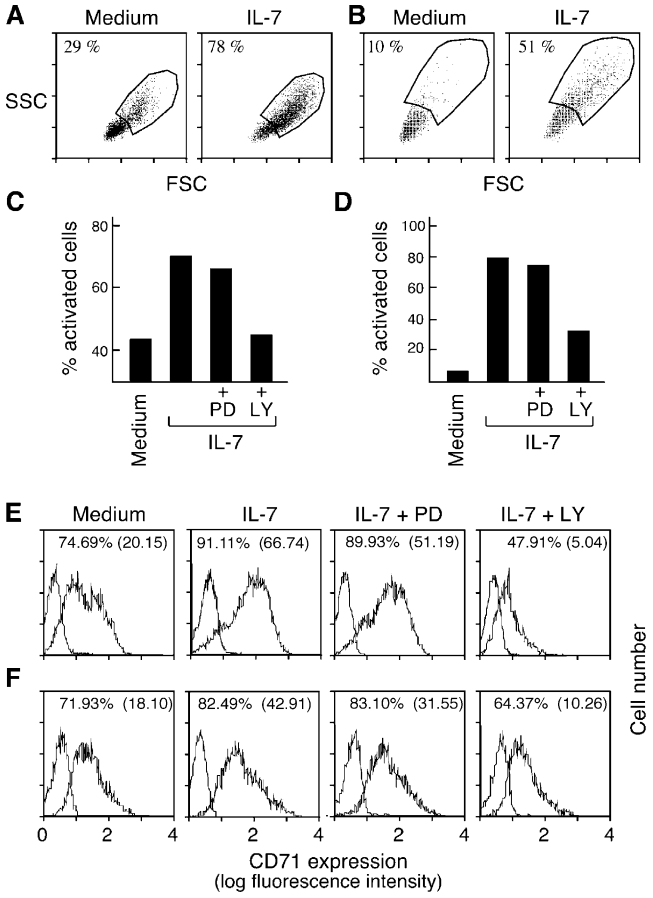

Figure 5. PI3K is critical for IL-7–mediated cell growth and activation of T-ALL cells. TAIL7 (A) and primary T-ALL (B) cells were cultured for 72 h in medium alone or with 10 ng/ml IL-7, and then analyzed by flow cytometry for changes in cell size (determined by FSC) in the live cell population. Percentage of "activated" cells was calculated by defining a threshold gate that excluded the bulk, small-sized population of medium-cultured cells. Results from one representative patient of five tested are shown in B. Results shown in A are similar to those found in numerous independent experiments. TAIL7 cells (C) and primary T-ALL cells (D) were cultured for 96 h under the indicated conditions, and the percentage of activated cells was calculated. TAIL7 cells cultured for 96 h (E) and primary T-ALL cells cultured for 72 h (F) under the indicated conditions were stained with anti-CD71 antibody. Results were expressed as the percentage of positive cells and as the specific MIF (in brackets). Results from primary T-ALL cells and the TAIL7 cell line were similar in three independent experiments.

(Fig. 5 A), consistently with previous results (8). Likewise, IL-7 increased cell size in all primary T-ALL samples (five cases analyzed; Fig. 5 B). To determine which intracellular pathways were involved in mediating IL-7–induced growth of T-ALL cells, we blocked PI3K–Akt and MEK–Erk pathways with the specific inhibitors. LY294002 completely inhibited or greatly impaired IL-7–mediated increase of cell size in TAIL7 (Fig. 5 C) and in primary T-ALL cells (Fig. 5 D). In contrast, PD98059 induced only a minor decrease in the percentage of activated cells.

Next, we used TAIL7 (Fig. 5 E) and primary T-ALL cells (Fig. 5 F) to compare the surface expression of activation markers CD71 and CD69 between medium- and IL-7–cultured cells. Flow cytometry analysis revealed that both CD71 (Fig. 5, E and F) and CD69 (not depicted) were strongly up-regulated by IL-7. Consistently with the effects on IL-7–mediated cell size increase, LY294002, but not PD985002, completely inhibited IL-7–mediated surface expression of CD71 in TAIL7 cells (Fig. 5 E) and primary T-ALL cells (Fig. 5 F), as assessed by both specific MIF and percentage of $CD71^+$ cells. Similar results were obtained with CD69 (not depicted). These results indicate that the MEK–Erk pathway does not significantly affect IL-7–mediated cell growth and activation of T-ALL cells, whereas PI3K-dependent signaling is critical for the regulation of these effects.

IL-7 Induces Glut1 Expression and Promotes Glucose Uptake by T-ALL Cells. Activation and growth of normal T cells is associated with increased glycolysis (28). Cytokines can induce expression of glucose transporters (29, 30) and augment glucose uptake (31) and glycolytic rates (32). The PI3K–Akt pathway is specifically involved in these processes in normal T lymphocytes (29). Because PI3K had a critical role in IL-7–mediated promotion of viability and induced cell growth and activation of T-ALL cells, we examined whether this correlated with the expression of the glucose transporter Glut1. As shown in Fig. 6 A, IL-7 up-regulated Glut1 in TAIL7 cells and this effect was dependent on PI3K activity because it was abrogated by the use of LY (Fig. 6 A). Subsequent to PI3K-dependent induction of Glut1 expression, IL-7 also increased glucose uptake by TAIL7 cells (Fig. 6 B). Thus, IL-7 provides the machinery for nutrient use by T-ALL cells and this effect is mediated in a PI3K-dependent manner.

IL-7 Regulates Mitochondrial Homeostasis in a PI3K-dependent Manner in T-ALL Cells. Glucose metabolism, similarly to antiapoptotic Bcl-2 family members, can maintain mitochondrial integrity and homeostasis (30), and consequently negatively regulate apoptosis (31, 32). In our system, IL-7 up-regulated Bcl-2, which is directly involved in regulation of mitochondrial integrity. In addition, IL-7 induced expression of Glut1, a regulator of glucose transport, which indirectly controls mitochondrial integrity (32). For these reasons, we analyzed the effect of IL-7 on mitochondrial homeostasis of TAIL7 cells. Mitochondrial homeostasis can be evaluated by measuring mitochondrial membrane potential ($_{\Delta}\psi_m$) using the potentiometric dye TMRE and flow cytometry analysis (30). Initial analyses of the whole cell population demonstrated that IL-7 up-regulated $_{\Delta}\psi_m$ in

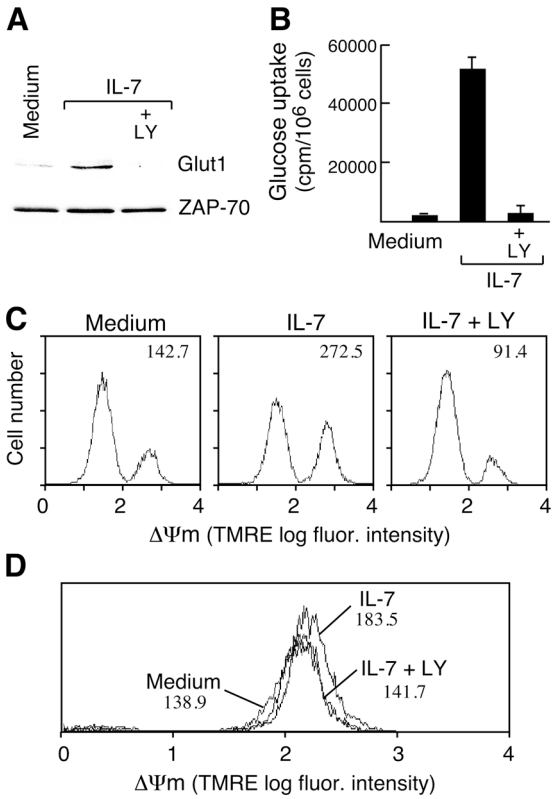

Figure 6. IL-7 regulates Glut1 expression and mitochondrial homeostasis via PI3K activation. TAIL7 cells were cultured for 96 h under the indicated conditions. (A) Cell lysates were resolved with 10% SDS-PAGE and immunoblotted with anti-Glut1 antibody. Anti–ZAP-70 antibody was used in the same membrane to ascertain even protein loading. (B) IL-7 promotes glucose uptake in a PI3K-dependent manner. TAIL7 cells were cultured as indicated for 96 h. Cells were then assayed for glucose uptake as described in Materials and Methods. (C) Mitochondrial membrane potential ($_{\Delta}\psi_m$) was assessed at 96 h of culture by staining TAIL7 cells with the potentiometric dye TMRE and analyzing the whole population by flow cytometry. TMRE intensity reflects the $_{\Delta}\psi_m$. Results are expressed as MIF. (D) Viable cells were identified and selected by flow cytometry by gating on the live cell population, as determined by FSC × SSC and/or annexin V–FITC staining. $_{\Delta}\psi_m$ of this population was determined using TMRE. Results are representative of three independent experiments.

TAIL7 cells, and LY294002 completely abrogated this effect (Fig. 6 C). The percentage of TMRE high cells (Fig. 6 C, right peak of the histograms) was similar to the percent of viable cells identified using annexin V/propidium iodide staining (not depicted). Because differences in $_{\Delta}\psi_m$ can be measured even before early manifestations of apoptosis (33), we next focused our analysis on the live population (Fig. 6 D). IL-7–mediated up-regulation of $_{\Delta}\psi_m$ on this population was abrogated by LY294002 (Fig. 6 D), suggesting that maintenance of mitochondrial integrity and homeostasis is an early event in the regulation of PI3K-dependent cell viability mediated by IL-7.

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 up-regulating Bcl-2 expression and viability, and also by inducing cell cycle progression (1, 33). In humans and mice, defective IL-7R expression results in severe T cell deficiency (34, 35), indicating that IL-7 plays an essential role during T cell ontogeny. Primary leukemic T cells show increased proliferation (2) and viability (3) when cultured with IL-7, suggesting that IL-7 might also be involved in the pathobiology of T-ALL. Up-regulation of cdk activity with consequent Rb hyperphosphorylation and progression toward S phase are absolutely dependent upon IL-7–induced down-regulation of $p27^{kip1}$. In addition, $p27^{kip1}$ down-regulation is associated with up-regulation of Bcl-2, which in turn is essential for IL-7–mediated survival of T-ALL cells (7).

Cytokines and growth factors can not only influence survival, but also cell growth through effects on glucose transporter expression, glucose uptake, and glycolysis (29, 30, 32). Lymphocytes require extrinsic stimulation to induce expression of surface receptors such as Glut1 that promote nutrient uptake and increase metabolic activity (36). TCR and IL-7R signals are among the signals that induce Glut1 in mature T cells. The importance of these extrinsic signals to promote nutrient intake in mature T cells was originally revealed by the observation that Bcl-2 transgene expression maintains cell survival, but does not prevent cell atrophy resulting from limited energy supplies due to inadequate nutrient uptake (36, 37). In this study we demonstrated that Glut1 is induced in high amounts in T-ALL cells by IL-7 and that its expression is dependent on IL-7–mediated signaling. In parallel with increased Glut1 expression, IL-7 also promoted nutrient uptake, increase of cell size, activation, and subsequent cell cycle progression and proliferation of T-ALL. Our results strongly suggest that IL-7 signals are needed not only to promote survival of leukemia cells by up-regulating Bcl-2, but also to provide the means for the generation of metabolic energy for initiating the cell cycle progression program that will eventually lead to cellular proliferation and expansion.

We examined the signaling pathways that might link IL-7 to the downstream regulators of viability and cell cycle, particularly to Bcl-2, Glut1, and $p27^{kip1}$. Knowledge regarding IL-7–mediated pathways in T cells is rather incomplete and very little is known about the integrity and biological role of those pathways in T-ALL cells. PI3K–Akt and MEK–Erk pathways have been associated with TCR- or cytokine-mediated expansion of T cell precursors and mature T cells (19, 20, 38). The PI3K–Akt pathway is activated by IL-7 in normal T cells (18, 23). In contrast, most studies with primary human mature T cells and murine T cell lines have shown that IL-7 does not mediate MEK–Erk activation (23), nor does it phosphorylate the MEK–Erk upstream molecules Shc (23, 39) and Ras (40). Our studies showed that in T-ALL cells, IL-7 activates Erk1/2 in a time- and dose-dependent manner that relies on MEK activity. However, inhibition of the MEK–Erk pathway does not affect IL-7–mediated viability or cell cycle progression of TAIL7 cells, indicating that these events occur in a MEK–Erk-independent manner. Studies in different cell types support an active role for Ras and MEK–Erk in $p27^{kip1}$ phosphorylation and consequent degradation by the ubiquitin–proteasome system (41). In T-ALL cells we found that down-regulation of $p27^{kip1}$ protein expression and Rb hyperphosphorylation that result from culture with IL-7 were not reverted by MEK inhibition. Hence, although the MEK–Erk pathway is activated by IL-7 in T-ALL cells, its exact biological role remains to be determined.

Our studies showed that IL-7 induced phosphorylation of Akt and its downstream targets GSK-3, FOXO1, and FOXO3a in a PI3K-dependent manner, indicating the existence of a functional IL-7–mediated PI3K–Akt pathway in T-ALL cells. Our subsequent studies with the PI3K inhibitor LY294002 demonstrated that activation of PI3K is mandatory for Bcl-2 up-regulation, Glut1 induction, glucose uptake, $p27^{kip1}$ down-regulation, and Rb hyperphosphorylation in IL-7–cultured T-ALL cells. Accordingly, IL-7 mediates cell cycle progression and viability of T-ALL cells via PI3K-dependent signals. Several studies have shown that engagement of the IL-7R induces activation of PI3K and PI(3,4,5)P3 production in human thymocytes, T lineage ALL blasts, and T-ALL cell lines (2, 40, 42), leading to their survival and proliferation (2, 18, 40). However, the exact PI3K-dependent mechanisms through which IL-7 exerts its effects in T cells are still under investigation. Although we cannot rule out the possibility that other PI3K downstream targets such as PKC or ILK (43, 44) might contribute to IL-7–induced functional outcomes, we favor the possibility that Akt is the main effector of IL-7–stimulated PI3K in T-ALL. First, IL-7 induced phosphorylation of FOXO1 and FOXO3a at threonine residues Thr24 and Thr32, which are targets for Akt kinase activity. Second, IL-7 induced phosphorylation of the Akt target GSK-3.

Phosphorylation of members of the Forkhead Box O (FOXO) family of transcription factors FOXO1, FOXO3a, and FOXO4 by Akt induces their inactivation and nuclear export (25). FasL and $p27^{kip1}$, which can be involved in apoptosis, are transcriptionally up-regulated by FOXO family members (25, 45). Thus, FOXO inactivation by the PI3K–Akt pathway may contribute to down-regulation of $p27^{kip1}$ by IL-7. Phosphorylation of GSK-3 results in its inactivation. Because active GSK-3 can mediate cell death, inactivation of GSK-3 by phosphorylation may promote cell viability (46). GSK-3β may also phosphorylate cyclin D1 (47) and c-Myc (48), promoting their protein degradation and contributing to cell cycle arrest. In addition, GSK-3 can also phosphorylate and inhibit NF-ATc, a transcription factor involved in proliferation (49, 50) and Bcl-2 gene transcription (51). Thus, GSK-3 phosphorylation and subsequent inactivation could result in up-regulation of Bcl-2 via activation of NF-ATc transcriptional activity (51) and down-regulation of $p27^{kip1}$ via c-Myc protein stabilization (48, 52).

A drop in $_{\Delta}\psi_{m}$ occurs very early during apoptosis (53). We showed that IL-7 up-regulates $_{\Delta}\psi_{m}$ in T-ALL cells in a

PI3K-dependent manner. This could be achieved via regulation of Bcl-2 expression (54, 55). Another possible mechanism may involve IL-7–mediated induction of glucose uptake and metabolism, which subsequently regulates mitochondrial homeostasis and $_{\Delta}\psi_{m}$. Consistently with the second mechanism, our results showed that IL-7 induced Glut1 glucose transporter expression and glucose uptake. Cytokine- or oncogene-induced glucose uptake appears to regulate mitochondrial homeostasis, thereby maintaining mitochondrial integrity and preventing apoptosis (31, 32). Conversely, glucose depletion or inhibition of glucose uptake is linked with cell death (31, 36). Here we showed that IL-7 up-regulates the expression of the glucose transporter Glut1 via PI3K activation. Thus, PI3K might control mitochondrial integrity and prevent apoptosis by regulating both Bcl-2 expression and glucose metabolism in T-ALL cells. Further studies are required to dissect the individual contribution of Bcl-2 and glucose metabolism in IL-7–regulated mitochondrial homeostasis.

Our studies have shown that IL-7–mediated up-regulation of Glut1 is associated with an increase in cell size. This finding may have significant implications on T-ALL pathobiology. Recent evidence suggests that there might be a correlation between increased cell size and oncogenesis (16). Moreover, tumor progression may not only depend upon uncontrolled cell cycle progression, but also upon unbalance of cell size regulatory mechanisms (56). Activation of lymphocytes is associated with increased size and metabolic activity (28, 36). The transferrin receptor CD71 is up-regulated by lymphocytes upon activation as a mechanism to meet the increased iron demands associated with increased metabolism (57). Increased Glut1 expression, glucose uptake, and glycolytic rates mediated by external signals allow T cells to anticipate energetic and biosynthetic needs associated with activation and cell growth (28). Our study showed that IL-7 contributes to T-ALL cell growth and activation, as shown by a dramatic increase in cell size and surface expression of CD71 and CD69, which correlate with induction of Glut1 expression. All of these events are dependent on PI3K activation. In mature T cells, the PI3K–Akt pathway regulates glucose metabolism mediated by CD28 costimulation (28), and Akt-controlled glucose uptake can promote survival and cell growth in other cell types (30, 58). Interestingly, in developing $CD8^{+}$ single positive thymocytes, IL-7 up-regulates Glut1 expression and glucose uptake (59).

There is mounting evidence that exogenous stimuli, particularly IL-7, may confer a selective advantage to leukemic T cells and play a fundamental role in leukemia pathophysiology (3, 6, 7, 60–62). Our studies presented here showed that IL-7–mediated activation of PI3K–Akt is not only essential for increased viability, but also critically involved in the regulation of metabolic activity, cell size, and proliferation of T-ALL cells, suggesting that this pathway may have an indispensable role in T-ALL biology. Importantly, overactivation of the PI3K–Akt pathway is associated with tumorigenesis (12, 16). Consistently, Jurkat and other T-ALL cell lines lack expression of PTEN, a phosphatase that targets PI(3,4,5,)P3, and consequently have high constitutive Akt activity (17). Taken together, our results support the conclusion that PI3K is a pivotal mediator of IL-7 signaling in T-ALL cells with a striking impact on several biological mechanisms necessary for tumorigenesis. These observations indicate that PI3K and its downstream targets might be essential for expansion of malignant T cells in vivo and may represent molecular targets for pharmacological intervention in T-ALL.

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