Thymic Stromal-Derived Lymphopoietin Induces Proliferation of Pre-B Leukemia and Antagonizes mTOR Inhibitors, Suggesting a Role for Interleukin-7Rα Signaling

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Abstract

Understanding the pathogenesis of leukemia in the context of lymphopoiesis may reveal novel therapeutic targets. Previously, we have shown that mTOR inhibitors (MTI) show activity *in vitro* and in preclinical models of both human and murine precursor B acute lymphoblastic leukemia (pre-B ALL), inhibiting cell proliferation and inducing apoptosis. These MTI-mediated effects can be reversed by interleukin-7 (IL-7), an important regulator of early B-cell development. This observation led us to examine the contribution of signaling via the IL-7Rα chain, which is shared by the receptor complexes of IL-7 and thymic stromal-derived lymphopoietin (TSLP). TSLP is closely related to IL-7 and active in lymphopoiesis, but an effect of TSLP on leukemia cells has not been described. We examined the effect of TSLP on pre-B ALL cells and their response to MTIs. Here, we show that TSLP stimulates proliferation of pre-B ALL cell lines. TSLP also partially reverses the effects of MTI on proliferation, apoptosis, and ribosomal protein S6 and 4E-BP1 phosphorylation in cell lines, with similar biological effects seen in some primary human lymphoblast samples. These data show that TSLP can promote survival of pre-B ALL cells and antagonize the effects of MTIs. These findings suggest that IL-7Rα chain is responsible for transducing the survival signal that overcomes MTI-mediated growth inhibition in pre-B ALL. Thus, further exploration of the IL-7Rα pathway may identify potential therapeutic targets in the treatment of ALL. Our data illustrate that growth-factor–mediated signaling may provide one mechanism of MTI resistance. [Cancer Res 2007;67(20):9963–70]

Introduction

Signal transduction inhibitors have provided important recent advances in cancer treatment. One such class of signal transduction inhibitors is the mTOR inhibitors (MTI), which include rapamycin and its derivatives. Although rapamycin is Food and Drug Administration approved as an immunosuppressive agent (1, 2), recent development of MTIs has focused on their use as anticancer signal transduction inhibitors (3). mTOR, which is a serine/threonine kinase, acts as a central regulator of cell growth and division, inducing cell cycle progression, cap-dependent translation, and ribosome synthesis (4, 5). It functions as a sensor to ensure that the cell is in an appropriate nutritional and energetic state before committing to cell growth and proliferation. Dysregulation and constitutive activation of the AKT/mTOR pathway leading to cell proliferation and survival have been shown in many different cancers (6). MTIs inhibit the growth of and/or induce apoptosis in many tumor types including lymphoid malignancies (7, 8). Previously, we have reported that MTIs induce growth inhibition and apoptosis of early B-cell progenitor acute lymphoblastic leukemia (pre-B ALL) cells; furthermore, these MTI-mediated inhibitory effects are reversed by the cytokine interleukin-7 (IL-7) (9, 10). Thus, we wanted to investigate further the mechanism by which signaling via the IL-7 receptor rescues rapamycin-treated pre-B ALL cells from growth inhibition to gain insight into the pathogenesis of ALL and its response to MTIs.

Cytokines, including IL-7 and thymic stromal-derived lymphopoietin (TSLP), are important regulators of normal lymphoid development and, therefore, may influence proliferation and/or survival of ALL cells (11–13). IL-7 and TSLP are closely related, yet distinct, in biological function. IL-7 is absolutely required for normal murine B- and T-cell development (14). IL-7 promotes the proliferation of pro–B cells, as well as developmental progression through the early B-cell stages (15, 16). Although IL-7 is absolutely required only for T-cell development in humans, it still plays an important role in human B-cell development, providing a survival signal for B lymphoid precursors (17, 18). In contrast to IL-7, information regarding TSLP and its function is limited. TSLP also supports early B-cell proliferation and development, but differentially affects murine B-cell progenitors of fetal and adult origin, acting earlier in the development of fetal-derived murine B cells (19–23).

The receptors and subsequent signaling pathways mediated by IL-7 and TSLP are also closely related: IL-7R and TSLPR are both heterodimeric complexes composed of two subunits, each including IL-7Rα chain and a partner subunit. In IL-7R, the partner subunit is the gamma common (γ_c) chain, (24) whereas in TSLPR, the partner is the TSLPR chain (25, 26). IL-7Rα has a much more restricted expression pattern than either γ_c or TSLPR (27). IL-7Rα is expressed from the early pro-B through the early pre-B stage of B-cell development and may be a marker for the common lymphoid progenitor (11, 28). Signal transduction through both IL-7R and TSLPR complexes ultimately results in the activation of signal transducer and activator of transcription (STAT) 5 and subsequent cell proliferation, but does so through different pathway intermediates: IL-7 signals through the Janus-activated kinase 1/3 (JAK1/3) kinases, whereas the TSLP-mediated signaling intermediates before STAT5 activation have not been fully elucidated (29–31).

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Although the role of IL-7 in normal lymphopoiesis has been extensively studied, the contribution of IL-7–mediated signaling in the development or progression of pre-B ALL has not been fully elucidated. However, there is evidence that IL-7 promotes survival of human T and B ALL cells, and that IL-7 has been associated with certain lymphoid malignancies (9, 32–34). Scupoli et al., found that IL-7 secreted by thymic epithelial cells protected T ALL cells from undergoing spontaneous apoptosis, and that this antiapoptotic effect was blocked by an anti-IL7Rα chain-specific monoclonal antibody (mAb; ref. 35). In contrast, a role of TSLP contributing to leukemogenesis has not been reported previously. Based on the similarities between IL-7 and TSLP, we hypothesized that TSLP would promote proliferation of pre-B ALL cells as well as rescue these cells from MTI-induced growth inhibition and cell death. Here, we report that TSLP acts very similarly to IL-7, stimulating proliferation of pre-B ALL cells and blocking the inhibitory effects induced by rapamycin, but with less potency. Because of the shared structure of IL-7R and TSLPR, these data suggest that signaling via the IL-7Rα chain may be responsible for transducing the signal that ultimately protects ALL cells from MTI-induced inhibitory effects.

Materials and Methods

Cells and culturing conditions. Eμ-RET transgenic mice have been previously described, and cell lines established from leukemic Eμ-RET transgenic mice were used in these studies (36). Immunophenotypically, these leukemia cell lines originate from either the pro-B (cytoplasmic μ−) or pre-B (cytoplasmic μ+) stage of B-cell development. These murine ALL cells were maintained at 37°C with 5% CO_2 in RPMI 1640 with L-glutamine, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 100 μmol/L nonessential amino acids, 100 units/mL penicillin, 100 μg/mL streptomycin (GibCo-BRL), 50 μmol/L 2-mercaptoethanol (Fisher Scientific), 10% FCS + 10% calf serum (HyClone; C20 media) with 2 ng/mL IL-7. Cell surface markers on these cells were determined by flow cytometry as described previously (36), and the phenotype was stable in culture over time (data not shown).

Cytokines and proliferation assays. Cells were cultured in IL-7–free C20 media for 24 h. For *in vitro* culture studies, 1 to 2×10^4 cells per well were cultured in triplicate in flat-bottomed 96-well plates with 0 to 100 ng/mL rapamycin (Calbiochem), 0 to 6 ng/mL recombinant mouse IL-7 (Leinco Technologies, Inc.), 0 to 30 ng/mL recombinant mouse TSLP (R&D Systems, Inc.), and 0 to 30 μg/mL α-IL-7Rα chain mAb (clone A7R34; eBioscience) for 3 to 5 days. Cell growth was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Co.) as previously described (37). Absorbance was measured at 570 nm using a BenchmarkPlus microplate spectrophotometer (Bio-Rad Laboratories). Results are expressed as mean of absolute absorbance number of treated sample divided by the mean of absolute absorbance number of the control sample. Results >1 indicates proliferation, whereas results <1 indicates growth inhibition.

Apoptosis assay. We plated 0.5 to 1×10^6 cells/mL in C20 media with 1 or 100 ng/mL rapamycin for 60 h. TSLP (30 ng/mL) was added to these cultures with a delay of 0 to 36 h after incubation with rapamycin. To detect apoptotic cells, levels of exposed phosphatidylserine on viable cells were measured using the ApoAlert Annexin V detection kit (BD Biosciences-Clontech) as previously described (38). Cells were incubated with FITC-conjugated Annexin V and 7-AAD (BD Biosciences-PharMingen) and analyzed by flow cytometry using a FACScalibur cytometer (Becton Dickinson Biosciences).

Cell lysates and immunoblotting. A total of 10^7 cells were incubated in C20 media with combinations of IL-7 (2 ng/mL), TSLP (30 ng/mL) and rapamycin (1 or 100 ng/mL) for 1, 2, or 6 h. After incubation, cells were washed with ice-cold PBS, and cell lysates were prepared as previously described (9, 10). Briefly, cells were resuspended in 100 μL ice-cold lysis buffer (Cell Signaling Technologies) containing Complete Mini (Roche Applied Biosciences) and 1 mmol/L phenylmethylsulfonyl fluoride (Sigma-Aldrich) and lysed by three freeze-thaw cycles. Forty μg of protein were loaded per lane and separated by electrophoresis on NuPage 10% or 4% to 12% gradient gels and transferred to polyvinylidene difluoride membranes (Invitrogen). Proteins were labeled with antibodies to the ribosomal protein S6, phosphorylated S6 [P-S6 ($Ser^{235/236}$)], 4E-BP1 total protein, phosphorylated 4E-BP1 [P-4E-BP1 (Thr^{70}); (Cell Signaling Technologies)], STAT5 and phosphorylated STAT5 (P-STAT5; Tyr694; Santa Cruz Biotechnology, Inc.) and then detected by chemiluminescence (Cell Signaling Technologies). Densitometric analysis was done using UnScanIt software (Silk Scientifics) to determine fold changes of phosphorylated proteins from treated relative to untreated lysates after normalization to corresponding total proteins.

Analysis of human primary ALL lymphoblasts using short-term bone marrow stromal layer culture system. Primary human lymphoblasts (obtained from the Leukemia Cell Bank, University of Pennsylvania, Philadelphia, PA) were cultured on fresh human bone marrow stromal cell layers (provided by Dr. John Choi, University of Pennsylvania, Philadelphia, PA) as previously described (10, 39, 40). After 20-Gy irradiation, human primary bone marrow stromal cells that were isolated from CD19-depleted normal donor bone marrow cells (10^5 per well) were seeded onto 24-well plates and cultured in C10 media (RPMI 1640 with L-glutamine, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 100 μmol/L nonessential amino acids, 100 units/mL penicillin, 100 μg/mL streptomycin, 50 μmol/L 2-mercaptoethanol + 10% FCS). After incubation overnight, cells were washed with serum-free X-vivo 10 media (Cambrex). For 48 h, primary human lymphoblasts (10^5 per well) were cultured with the stromal cells in X-vivo 10 media supplemented with 5% fetal bovine serum, SCF (20 mg/mL), insulin-like growth factor-I (20 ng/mL), and FLT-3 (10 ng/mL; R&D Systems, Inc.) containing rapamycin and TSLP (39). Viability was measured by visualization of cell cultures, enumeration of live cells by trypan blue exclusion, and flow cytometry for FSC/SSC and Annexin V/7-AAD positivity (BD Biosciences-PharMingen).

Statistics. Proliferation and apoptosis assays were analyzed by ANOVA with Dunnett's or Tukey's LSD comparison and the paired Student's *t* test using Analyse-It Software for Microsoft Excel, v. 1.73. A P value of <0.05 was considered significant.

Results

TSLP stimulates proliferation of ALL cell lines and the phosphorylation of STAT5. Because TSLP is a cytokine active in B-cell lymphopoiesis, we hypothesized that TSLP acts as a growth factor for pre-B ALL cells, supporting the importance of signaling via the IL-7Rα chain. If signaling via the γ_c chain of the IL-7R were vital to transmitting this survival response, then we would predict that IL-7 would have effects not seen with TSLP, whereas if signaling via the IL7-Rα were key in producing this response, then both cytokines would have similar effects. To evaluate the effects of TSLP on pre-B ALL, we used the murine early progenitor B (pre-B ALL) cell lines, T309, 289, 420.2, and 781, derived from Eμ-ret transgenic mice (9). These cell lines were cultured with increasing concentrations of TSLP (0–30 ng/mL). We chose this range because TSLP stimulates BA/F3 cells transfected with IL-7Rα chain within this range of TSLP concentration (26). After 4 days, cell proliferation was assessed using MTT. Figure 1*A* shows a dose-dependent proliferation in response to TSLP, which is similar to that seen with IL-7 (9). These pre-B ALL cell lines all showed a significant ($P < 0.0001$) proliferative response to TSLP, ranging from 1.5× (cell line 289) to almost 3× increase (cell line T309) in proliferation. Proliferation seemed to plateau at a concentration of 3 ng/mL TSLP for the previously identified IL-7–independent cell lines (289 and 420.2), but not the dependent cell lines (T309 and 781). In comparison, IL-7–stimulated proliferation plateaued at ~2 ng/mL (data not shown and ref. 9). Overall, response of each cell line to TSLP paralleled the response to IL-7 in that TSLP could

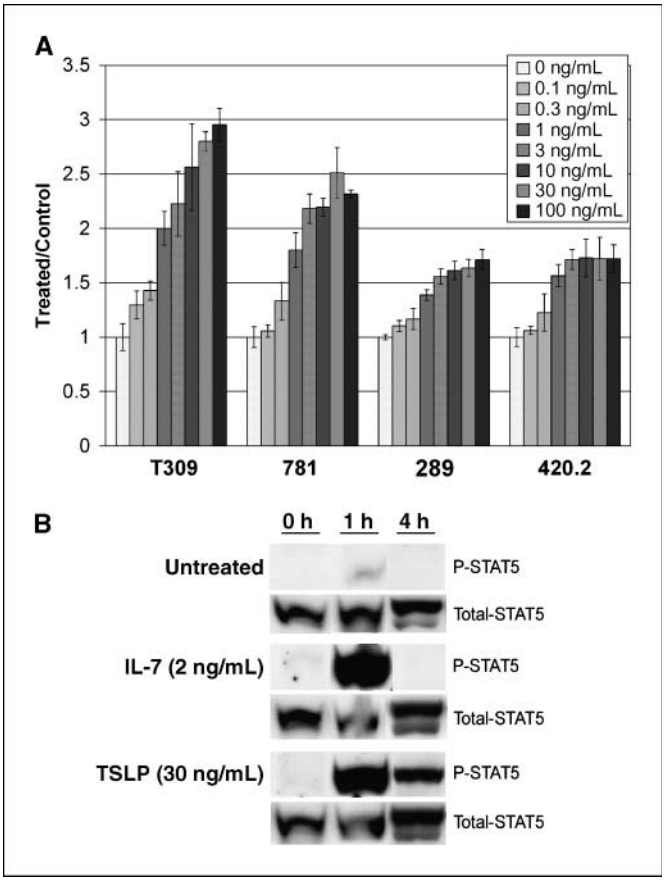

Figure 1. TSLP stimulates proliferation and STAT5 phosphorylation of ALL cell lines. *A,* TSLP induces cell proliferation. Eμ-ret transgenic mouse-derived pre-B ALL cell lines T309, 781, 289, and 420.2 were cultured with TSLP (0–30 ng/mL). Cells were incubated for 4 d, and then cell proliferation was assessed using MTT. All measurements were done in triplicate. *Columns,* mean of the treated/control (untreated) cells (relative absorbance of triplicate cultures, as described in Materials and Methods); *bars,* SE. Results from one of three independent experiments that were done on all four cell lines are presented here. Difference in proliferation as compared with untreated cultures was significant at TSLP doses $\geq$0.3 ng/mL for cell line 781 and $\geq$1 ng/mL for cell lines T309, 289, and 420.2 [one-way between subjects (BS) ANOVA with Dunnett's LSD comparison, $P < 0.0001$]. *B,* treatment with TSLP increases phosphorylation of STAT5 over time. After 24 h incubation in IL-7– and TSLP-free media, line 289 cells were cultured with IL-7 (2 ng/mL) or TSLP (30 ng/mL) for 0, 1, and 4 h. Cell lysates were analyzed by immunoblotting for phosphorylated and total STAT5 proteins (P-STAT5 and Total-STAT5, respectively). Each lane was loaded with equal amounts of protein. Results from one of two independent experiments are presented here.

replace IL-7 for all tested IL-7–dependent cell lines, as well as increase the proliferation of the IL-7–independent, IL-7–responsive cell lines. Coculture with both IL-7 and TSLP did not produce an additive, synergistic, or antagonistic effect (data not shown). These data show that both TSLP and IL-7 stimulate cell proliferation of ALL cells, supporting a role for IL-7Rα signaling in pre-B ALL.

Because activation of TSLP- and IL-7–mediated signal transduction pathways leads to the activation of the transcription factor STAT5 (41, 42) and because constitutive activation of the JAK/STAT pathway has been associated with a subset of hematologic malignancies (reviewed in ref. 43), we evaluated the phosphorylation status of STAT5 following stimulation with TSLP and IL-7 in pre-B ALL cells. After line 289 cells were stimulated with IL-7 or TSLP for 0 to 4 h, P-STAT5 along with total STAT5 proteins were detected via immunoblotting. Doses of IL-7 and TSLP (2 and 30 ng/mL, respectively) that produced maximal proliferative effect in culture were used. Although untreated cells had virtually no P-STAT5 detected, cells stimulated with either IL-7 or TSLP yielded increased phosphorylation of STAT5 over time (Fig. 1*B*). Although treatment with both cytokines resulted in STAT5 phosphorylation, they did so with different timing. Treatment with IL-7 for 1 h resulted in a substantial degree of STAT5 phosphorylation, but by 4 h, the level of P-STAT5 was back to baseline. In comparison, treatment with TSLP for 1 h increased the amount of P-STAT5 detected as compared with 0 h, but to a lesser extent as that observed with IL-7. However, there was still evidence of STAT5 activation after 4 h of treatment with TSLP. Although changes in P-STAT5 were observed, there was no significant change in total STAT5 proteins detected over time.

TSLP prevents growth inhibition and apoptosis in rapamycin-treated ALL cells. Given the similar proliferative responses, we evaluated the effect of TSLP on rapamycin-treated pre-B ALL cells. We found that TSLP was capable of rescuing murine pre-B ALL cell lines from rapamycin-induced growth inhibition (Fig. 2*A*). Although it did not rescue pre-B ALL cells from rapamycin-induced growth inhibition to the same extent as IL-7, TSLP had a significant effect ($P < 0.001$) on proliferation for each dose of rapamycin. TSLP (3–30 ng/mL) almost completely restored cell proliferation inhibited by low-dose rapamycin (1 ng/mL) from 50% up to 85–90% of baseline, untreated cell growth. At higher doses of rapamycin (10–100 ng/mL), TSLP only partially restored cell proliferation from 20% to only 50–60% of baseline, a difference that was still statistically significant ($P = 0.003$). In comparison, IL-7 almost completely reversed cells from rapamycin-induced growth inhibition even at high doses of rapamycin (100 ng/mL). Again, treatment with IL-7 and TSLP concurrently did not have an additive effect on rescuing rapamycin-treated ALL cells (data not shown).

It was important to show that the reversal of MTI-induced growth inhibition was due to the prevention of apoptosis and cell death by the cytokines rather than an outgrowth of a subpopulation of MTI-resistant, cytokine-responsive cells. To test this, we treated pre-B ALL cells with rapamycin (1 and 100 ng/mL) and then added 30 ng/mL TSLP from 0 to 48 h later. After 60 h (total in culture), the extent of apoptosis and cell death was determined using staining for Annexin V (to detect apoptotic cells) and 7-AAD (to detect nonviable cells). Rapamycin induced apoptosis and cell death in a dose-dependent manner, with significantly more (by ~2.5-fold) apoptotic and dead cells seen at the high dose versus low dose of rapamycin (Fig. 2*B* and *C*). TSLP impacted the cell viability of these rapamycin-treated pre-B ALL cells in a time-dependent manner. TSLP almost completely prevented apoptosis in culture conditions in which cells were concomitantly exposed to TSLP and either dose of rapamycin. A delay of 12 and 24 h in adding TSLP also resulted in a significant ($P = 0.002$) reversal of rapamycin-induced apoptosis. However, a delay of TSLP addition beyond 24 h impaired the ability of TSLP to prevent apoptosis, with TSLP having very little effect on apoptosis after 36 to 48 h of rapamycin exposure. Similar results were seen with delayed administration of IL-7 (data not shown). These results indicate that both TSLP and IL-7 block rapamycin-induced apoptosis and cell death within ALL cells as opposed to promoting rapamycin-resistant cell proliferation.

IL-7 and TSLP induce phosphorylation of the mTOR pathway intermediates S6 and 4E-BP1, an effect antagonized by rapamycin. To further evaluate the mechanism by which TSLP-mediated signaling interacts with the mTOR pathway, we used

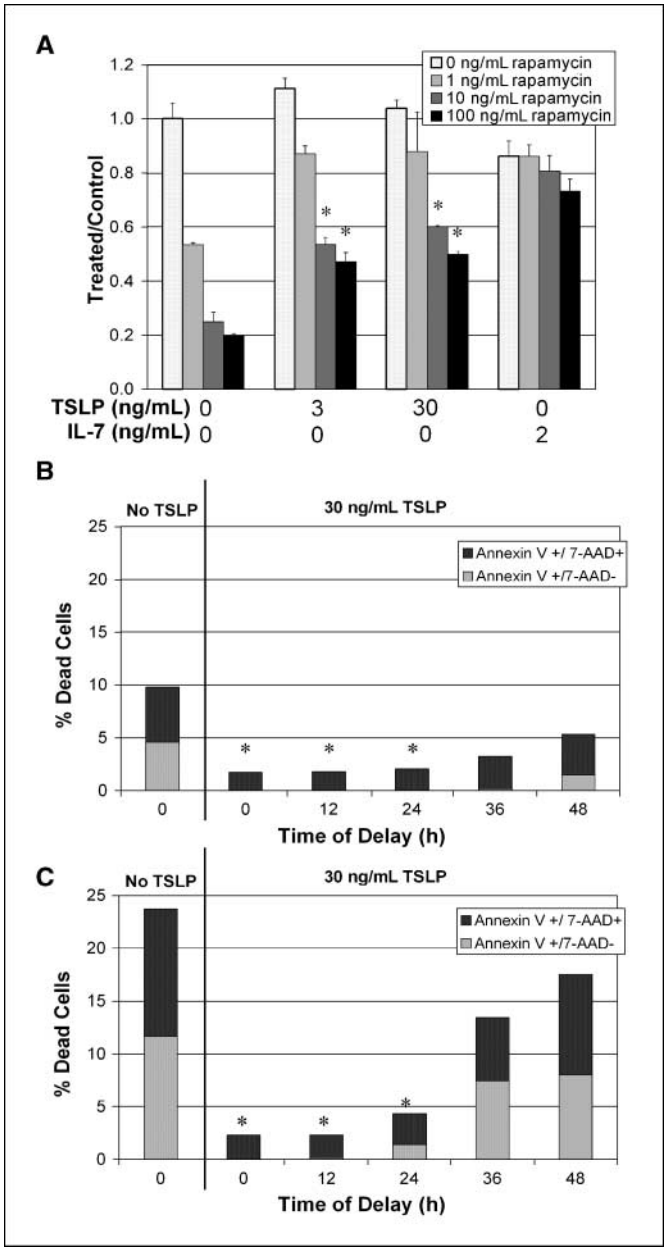

Figure 2. TSLP rescues rapamycin-treated ALL cells from growth inhibition, apoptosis, and cell death. *A,* cells were cultured with combinations of rapamycin (0–100 ng/mL), TSLP (0–30 ng/mL), and IL-7 (0–2 ng/mL). After 3 d of incubation, cell proliferation was assessed by using MTT. All measurements were done in triplicate. *Columns,* mean of the treated/control (untreated) cells (relative absorbance of triplicate cultures, as described in Materials and Methods); *bars,* SE. Results of one of at least three independent experiments are presented here. Similar results were found with other IL-7–independent and IL-7–dependent cell lines (data not shown). For each dose of rapamycin, TSLP has a significant effect on proliferation as compared with rapamycin alone (two-way BS ANOVA, $P < 0.0001$). *, significant differences of TSLP reversal of growth inhibition induced by high doses (10 and 100 ng/mL) of rapamycin as compared with control (two-way BS ANOVA with Tukey's LSD comparison, $P = 0.003$). *B* and *C,* cells were cultured with 1 ng/mL (*B*) or 100 ng/mL (*C*) rapamycin for 60 h. TSLP (30 ng/mL) was added with a delay of 0 to 48 h. Apoptosis and cell death were assessed by labeling with FITC-conjugated Annexin V and 7-AAD and then analyzed by flow cytometry. Total bar represents % apoptotic and dead cells, with the bottom portion of each bar representing % Annexin V single-positive cells and top portion representing % Annexin V/7-AAD double-positive cells. These data represent one of two independent experiments. Similar results were seen with cell line 420.2 (data not shown). *, significant differences of % dead cells in rapamycin-treated cells with 0, 12, or 24 h delay of TSLP administration as compared with cells treated for 60 h with rapamycin alone (paired samples Student's *t* test, $P = 0.002$).

immunoblotting of ALL cell lysates to detect post-translational modifications of ribosomal S6 and 4E-BP1, two mTOR pathway intermediates. After treatment with combinations of IL-7, TSLP, and rapamycin for 60 and 120 min, phosphorylated S6 (P-S6) ($Ser^{235/236}$) and total S6 proteins were detected. Over time, treatment with either IL-7 or TSLP increased S6 phosphorylation (Fig. 3*A*). As compared with untreated cultures, IL-7 induced a 3- and 4-fold increase in S6 phosphorylation after 60 and 120 min of incubation, respectively. TSLP treatment also resulted in S6 phosphorylation but was more delayed: at 60 min, treatment with TSLP resulted in only a modest 20% increase, but by 120 min, TSLP and IL-7 induced a comparable degree of S6 phosphorylation. In contrast, treatment with both low- and high-dose rapamycin resulted in profound hypophosphorylation of S6. Addition of either IL-7 or TSLP partially restored the phosphorylation of S6 in the presence of rapamycin in a dose-dependent manner.

Another downstream target of mTOR, 4E-BP1, was evaluated after 6 h of treatment with combinations of TSLP, IL-7, and rapamycin. Detection of P-4E-BP1 yielded similar results as P-S6: when normalized to total 4E-BP1 proteins, treatment with

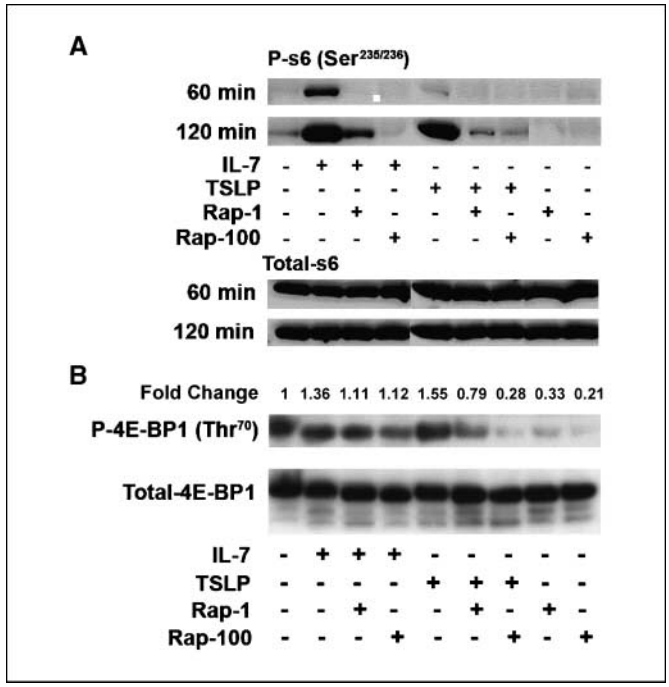

Figure 3. IL-7 and TSLP induce phosphorylation of S6 and 4E-BP1, whereas rapamycin abrogates this effect over time. Line 289 cells were cultured with combinations of IL-7 (2 ng/mL), TSLP (30 ng/mL), and rapamycin at 1 ng/mL (Rap-1) or 100 ng/mL (Rap-100) for 60 and 120 min and 6 h. Each lane was loaded with 40 μg of protein. Results from one of two independent experiments are presented here. Using UnScanIt software, densitometric analysis was done to quantitate changes in band intensity relative to untreated cultures. *A, top,* immunoblots of P-S6 ($Ser^{235/236}$) after treatment for 60 and 120 min. When normalized to total S6 proteins, 60-min treatment with IL-7 and TSLP resulted in a 3- and 1.2- fold increase in P-S6 band intensity, respectively; 120-min treatment with IL-7 and TSLP resulted in a >3.5-fold increase. Relative to treatment with rapamycin alone, IL-7 with 1 and 100 ng/mL rapamycin restored S6 phosphorylation by 50% and 25%, respectively, and TSLP with 1 and 100 ng/mL rapamycin resulted in a 35% and 24% increase in P-S6, respectively. *Bottom,* immunoblots of total ribosomal protein S6 (Total-S6). There was no significant change in total S6 protein under all treatment conditions. *B,* immunoblots of phosphorylated 4E-BP1 (P-4E-BP1(Thr^{70})) and total 4E-BP1 after treatment for 6 h. Densitometric measurement of relative changes in P-4E-BP1(Thr^{70}) was done after normalization against total 4E-BP1 protein. Fold change values >1 denote increased phosphorylation and <1 denote decreased phosphorylation as compared with untreated cultures.

IL-7 or TSLP resulted in a 30% to 50% increase in P-4E-BP1 as compared with untreated cultures (Fig. 3*B*). Treatment with 1 and 100 ng/mL rapamycin caused profound hypophosphorylation of 4E-BP-1, with a 3- and almost 5-fold decrease in band intensity, respectively. IL-7 and TSLP restored phosphorylation of 4E-BP1 in rapamycin-treated cells. Again, the reversal seen with IL-7 was more complete than that observed with TSLP. Taken together, these results show that signaling mediated by TSLP and IL-7 interferes with the inhibitory actions of MTIs in ALL cells.

The antagonistic anti–IL-7Rα chain mAb A7R34 inhibits cell proliferation of IL-7– and TSLP-responsive ALL lines and blocks the ability of IL-7 and TSLP to reverse mTOR inhibition. To further study the role of IL-7Rα chain signaling in pre-B ALL, we used an antagonistic anti–IL-7Rα mAb (A7R34; ref. 28, 35). We evaluated the effects of this α-IL-7Rα mAb on both cytokine-dependent (T309) and -independent (289) cell lines. Cells were cultured with combinations of IL-7, TSLP, rapamycin, and 0 to 30 μg/mL α-IL-7Rα mAb. After 3 days incubation, cell proliferation was assessed using MTT. In the absence of IL-7 or TSLP in the culture media, incubation with the antibody alone showed no significant effect on cell proliferation ($P = 0.15$). Addition of TSLP at 3 and 30 ng/mL significantly increased proliferation, consistent with the results above ($P = 0.0002$; see Fig. 4*A*). This proliferative effect was inhibited substantially, but not completely, by the addition of α-IL-7Rα mAb ($P = 0.014$). Similarly, IL-7 at 0.2 and 2 ng/mL increased proliferation as compared with untreated cells ($P = 0.002$; Fig. 4*B*). However, we observed that IL-7–induced proliferation was completely inhibited by ≥1 μg/mL α-IL-7Rα mAb, with no statistically significant difference in proliferation between cells treated with IL-7 + α-IL-7Rα mAb as compared with α-IL-7Rα mAb alone ($P = 0.89$).

As seen in Fig. 4*C*, 100 ng/mL rapamycin significantly inhibited ($P < 0.0001$) cell proliferation; addition of α-IL-7Rα mAb had no further significant effect ($P = 0.52$). Again, IL-7 completely and TSLP partially reversed rapamycin-induced inhibition. Here, the presence of α-IL-7Rα mAb significantly antagonized the rescue of rapamycin-treated cells by either IL-7 or TSLP ($P = 0.0007$ and $P = 0.001$, respectively). The effect of α-IL-7Rα mAb was more extensive on IL-7- than TSLP-treated cells. With doses ≥1 μg/mL of α-IL-7Rα mAb, there was complete blockade of the ability of IL-7 to reverse rapamycin, with a lesser but still statistically significant ($P = 0.001$) antagonism seen in the TSLP + rapamycin cultures. This modest antagonism of TSLP by α-IL-7Rα mAb may reflect the ability of TSLP to only partially rescue cells from rapamycin-induced inhibition. Although IL-7 was more sensitive than TSLP, the ability of both cytokines to induce proliferation and reverse rapamycin-induced inhibition could be antagonized by specifically blocking the actions of the IL-7Rα chain.

Biological effects of TSLP on primary human ALL lymphoblasts. To evaluate the effect of TSLP on primary human lymphoblasts, we examined cell viability by cell enumeration and morphologic assessment. In addition, the extent of apoptosis and cell death was determined by Annexin V and 7-AAD staining, as above. Primary lymphoblasts were obtained from four different patients with pre-B ALL. Their demographics, immunophenotypes, and cytogenetics are summarized in Table 1. A total of 1×10^5 primary human ALL cells per well were cultured on normal bone marrow stromal layers and exposed to low-dose rapamycin (1 ng/mL) and TSLP (30 ng/mL). Cells treated with rapamycin alone had a 24-fold decrease in viable cells as compared with control wells, from 1.2 to 0.05×10^6 cells. In contrast, TSLP-treated

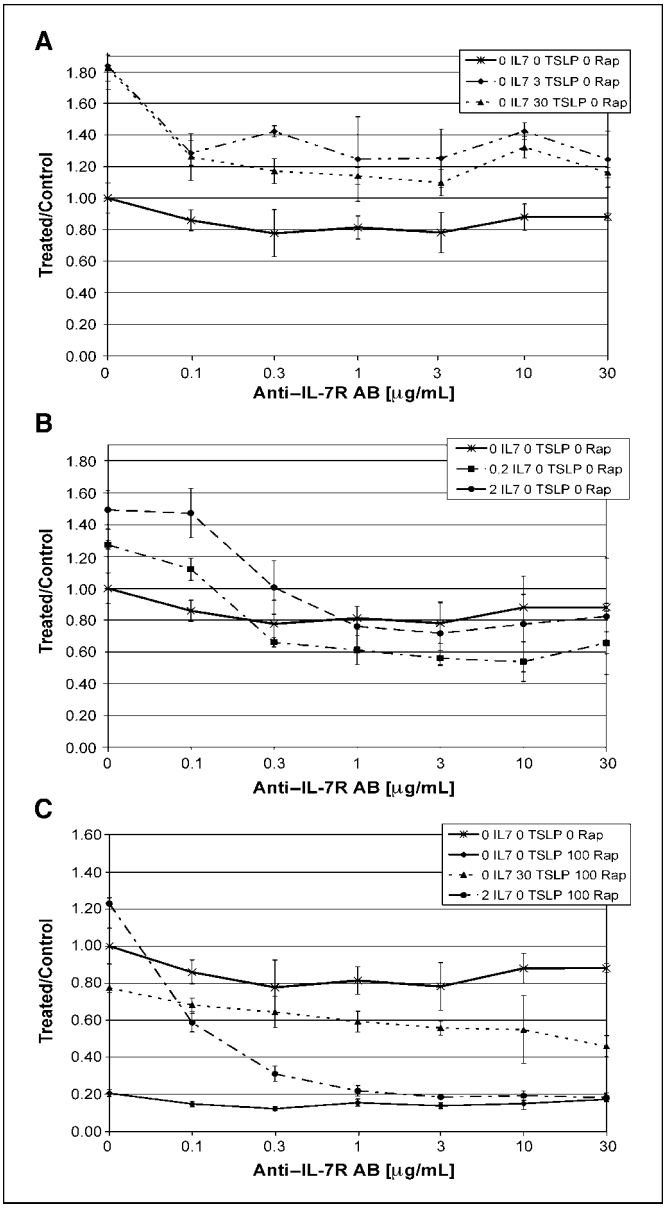

Figure 4. Antagonistic α-IL-7Rα mAb can block the proliferative effects of IL-7 and TSLP on pre-B ALL cells. Line T309 cells were cultured with combinations of IL-7, TSLP, rapamycin, and α-IL-7Rα mAb (0–30 μg/mL) for 3 d. Cell proliferation was assessed by MTT. All measurements were done in triplicate. *Points,* mean of the treated/control (untreated) cells; *bars,* SE. Results of one of three independent experiments are presented here. There was no statistical difference in proliferation with increasing concentrations of α-IL-7Rα mAb. *A,* cells were cultured with combinations of 3 or 30 ng/mL TSLP and increasing concentrations of α-IL-7Rα mAb. TSLP caused significant differences in increased proliferation, with a 1.3- to 1.5-fold increase above baseline, untreated cultures (one-way BS ANOVA with Dunnett's LSD comparison, $P = 0.0002$); the presence of increasing concentrations of α-IL-7Rα mAb led to a significant reduction in TSLP-induced proliferation by 1.5- to 1.6-fold (two-way BS ANOVA, $P = 0.014$). *B,* cells were cultured with combinations of 0.2 or 2 ng/mL IL-7 and increasing concentrations of α-IL-7Rα mAb. IL-7 alone caused significant differences in increased proliferation by 1.3- to 1.5-fold (one-way BS ANOVA with Dunnett's LSD comparison, $P = 0.002$); and the presence of increasing concentrations of α-IL-7Rα mAb caused a significant reduction in IL-7–induced proliferation (two-way BS ANOVA, $P = 0.007$). *C,* cells were cultured with combinations of 2 ng/mL IL-7, 30 ng/mL TSLP, 100 ng/mL rapamycin, and increasing concentrations of α-IL-7Rα mAb. Rapamycin alone decreased proliferation significantly (two-way BS ANOVA, $P < 0.0001$); addition of α-IL-7Rα mAb to rapamycin-treated cells produced no significant difference in proliferation (two-way BS ANOVA, $P = 0.52$), but it induced a statistically significant reduction in IL-7 rescue (two-way BS ANOVA, $P = 0.0007$) and in TSLP rescue (one-way BS ANOVA with Tukey's LSD comparison, $P = 0.001$).

Table 1. Characteristics of human primary ALL lymphoblasts

Sample ID	Age (y)	Sex	Race	% lymphoblasts		Immunophenotype	Cytogenetics	Diagnosis
				BM	CBC			
96	N/A*	Female	Caucasian	N/A*	64	$CD19^+$, $CD10^+$, $CD34^+$, $sIgM^+$, TdT N/A*, $CD2^-$, $CD5^-$, $CD7^-$	46, XX	pre-B ALL, relapsed
195	28	Male	African-American	N/A*	95	$CD19^+$, $CD10^+$, $CD34^+$ (subset), $sIgM^-$, TdT^+, $CD2^-$, $CD5^-$, $CD7^-$	46, XY	pre-B ALL
359	42	Female	African-American	99	89	$CD19^+$, $CD10^+$, $CD34^+$ (dim), $sIgM^-$, TdT^+, $CD2^-$, $CD5^-$, $CD7^-$	Complex karyotype with 41% (+)BCR/ABL	pre-B ALL
3045	7	Male	Hispanic	83	76	$CD19^+$, $CD10^+$ (dim), $CD34^+$, $sIgM^-$, TdT^+, $CD2^-$, $CD5^-$, $CD7^-$	46, XY, t(l,9) del9p; del6q	pre-B ALL

*Not available.

cells showed an increase in cell density with complete confluence and areas of overgrowth as compared with untreated cells and a viable cell count of 1.9×10^6 cells per well. Furthermore, the addition of TSLP rescued the cells from rapamycin-induced cell death, resulting in 1.1×10^6 viable cells per well, which is comparable to the viability of untreated cells. The morphology of the cells exposed to both TSLP and rapamycin was similar to that of the untreated and TSLP-treated cells, but grew at a lower density. As seen in Fig. 5, although only 19% of untreated cells were undergoing apoptosis and cell death, 80% of rapamycin-treated cells were nonviable. Cells cocultured with rapamycin and TSLP after 48 h yielded only 25% nonviable cells, with 30% of cells treated with TSLP alone undergoing apoptosis and cell death. We observed that the lymphoblasts within these sample wells were found to be densely populated and potentially overgrown in response to TSLP, possibly accounting for the higher-than-expected rate of cell death in cell cultures treated with TSLP alone as compared with the background level of cell death in untreated cell cultures. Similar results yielding comparable changes in the percentage of viable versus nonviable cells after treatment with combinations of rapamycin, TSLP, and IL-7 were found in two (patient samples 96 and 195) out of four primary human samples tested (data not shown).

Discussion

Great strides have been made in the treatment of ALL in children over the past several decades, but for adult or high-risk patients

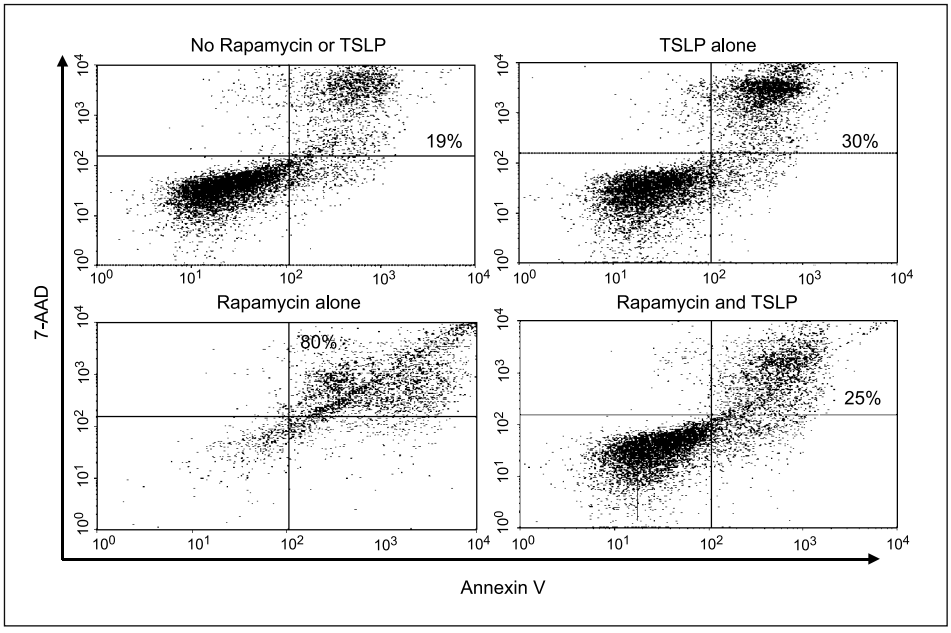

Figure 5. TSLP rescues some primary human ALL lymphoblasts from rapamycin-induced apoptosis and cell death. Lymphoblasts were cultured on bone marrow stromal cell layers and with media containing combinations of rapamycin (1 ng/mL) and TSLP (30 ng/mL). After 48 h of culture, apoptosis and cell death were assessed by flow cytometry. Lymphoblasts were labeled with FITC-conjugated Annexin V (*x*-axis) and 7-AAD (*y*-axis). One of four different patient samples tested in two independent experiments is presented here.

and for those who relapse, current treatment is usually unsuccessful. These patients have a poor prognosis despite aggressive multimodality therapies and may benefit from biologically targeted agents. Our investigations have focused on the effect of the MTI rapamycin, on pre-B ALL cells and its effect in response to cytokines that mediate survival signals during normal B-cell development. Previously, we have shown that rapamycin inhibits proliferation and induces apoptosis of pre-B ALL, effects which are reversed by IL-7 (9, 10). In addition, rapamycin as a single agent shows efficacy in mouse models of pre-B ALL (9, 10). Here, we report the response of pre-B ALL cells to TSLP, another important lymphopoietic cytokine. Specifically, we show that TSLP stimulates the proliferation of pre-B ALL cell lines and some primary human lymphoblasts *in vitro*, and that TSLP is capable of rescuing ALL cells from rapamycin-induced growth inhibition and apoptosis. Even with delayed administration, TSLP and IL-7 retain the capacity to suppress rapamycin-induced apoptosis for a period of up to 24 h, although not later. The most likely explanation for these results is that although MTI treatment eventually leads to triggering an irreversible pathway of apoptosis and cell death, commitment to apoptosis is not an early event, and survival pathways driven by these cytokines can initially overcome the effects of mTOR inhibition. The ability of these cytokines to induce STAT5 phosphorylation as well as prevent rapamycin-induced down-regulation of two downstream targets of mTOR, (S6 and 4E-BP1) supports this notion as well. These results implicate the mTOR pathway as a downstream mediator of TSLP and IL-7 signaling. In addition, we found that the mAb A7R34, which specifically blocks the actions of IL-7Rα chain, antagonizes the proliferative actions of both IL-7 and TSLP. Because IL-7R and TSLPR share the IL-7α chain, but not the γ_c chain, our results support the hypothesis that IL-7Rα signaling is a major contributor to the ability of these cytokines to antagonize the effects of rapamycin.

Although TSLP rescued pre-B ALL cells from growth inhibition and apoptosis induced by low-dose rapamycin (1 ng/mL), it only partially reversed this inhibition at higher doses of rapamycin. In comparison, IL-7 reversed pre-B ALL cell proliferation almost completely even in cells treated with high-dose rapamycin. Although blocking of the IL-7Rα chain in IL-7– and TSLP-treated cells antagonized the proliferative effects of these cytokines, this blockade was more effective in the case of IL-7 than TSLP. IL-7–mediated signaling quickly resulted in STAT5 phosphorylation, while TSLP also induced STAT5 phosphorylation, albeit with somewhat slower kinetics. We found similar results in the differential timing of S6 phosphorylation induced by IL-7 as compared with TSLP. In addition, as compared with TSLP, IL-7 was more effective at maintaining the phosphorylation of 4E-BP1 in the presence of rapamycin. These findings are consistent with the findings of Levin et al. (21), who studied the effect of TSLP on the pre-B cell line NAG 8/7. They found that NAG 8/7 cells respond more potently to IL-7 than to TSLP, and that both TSLP- and IL-7–driven proliferation is blocked only by the addition of anti–IL-7Rα chain-specific antibodies. Thus, in both systems, IL-7 seems to act as a more potent inducer of proliferation despite the common signaling events activated by both cytokines. These data suggest that, although IL-7Rα signaling is sufficient to produce the growth effects and impact on MTI response we observed in ALL, other components of the signaling complexes may potentiate or modulate this response.

Although the actions of IL-7 and TSLP are tightly integrated, their activities affect lymphopoiesis differently (44). The relationship between TSLP and IL-7 and their contribution to leukemic cell signaling may stem from differences in their receptor complexes. Transgenic mice that lack expression of IL-7Rα chain gene (IL-7R$^{-/-}$) result in a more severe defect in B-cell development as compared with mice that do not express the IL-7 gene (IL-7$^{-/-}$) because the loss of IL-7Rα chain signaling abrogates both IL-7– and TSLP-mediated signaling (45, 46). In contrast, mice lacking TSLPR subunit gene expression (TSLPR$^{-/-}$) seem to have a normally developed B-cell compartment in their bone marrow (47). The presence of the IL-7Rα chain significantly increases the binding affinity of TSLP to the TSLPR subunit alone, and cells coexpressing TSLPR, IL-7Rα, and γ_c chains proliferate to a greater extent in response to IL-7 as compared with TSLP (26). These data suggest that response to these cytokines may reflect the stoichiometry of the TSLPR and IL-7Rα subunits.

Mice that overexpress IL-7 (IL-7$^{+/+}$) have increased numbers of B and T cells and develop lymphomas (48), which do not occur in an IL-7Rα$^{-/-}$ background. A similar effect can be seen with alterations in STAT5 signaling, in that mitigation of STAT5 signaling leads to the prevention or delay in lymphomagenesis in IL-7$^{+/+}$ mice (49). These results support the notion that signal strength induced by IL-7 and TSLP and composition of the receptors transmitting this signal influence the survival of malignant cells and may account for the differences we observed in cell proliferation, survival, and response to MTI. The pairing of IL-7Rα with either the γ_c chain or TSLPR may also activate differentially other survival pathways within our murine pre-B ALL cells, resulting in the apparent difference in potency of the IL-7 versus the TSLP response to mTOR inhibition and blockade of the IL-7Rα chain in pre-B ALL cells.

In summary, we have shown an effect of the cytokine TSLP on pre-B ALL cells. This is the first report that we are aware of showing the proliferative effects of TSLP on pre-B ALL, including some, but not all, human primary pre-B lymphoblasts tested. TSLP can also rescue MTI-treated pre-B ALL cells from growth inhibition and apoptosis. Because of the similarities shared by the TSLPR and IL-7R signal transduction pathways, our data suggest that an essential component of the survival signal in these ALL cells is mediated by the IL-7Rα chain. Further investigation is needed to fully elucidate the interactions of the mTOR and IL-7–/TSLP-mediated signaling pathways in ALL cells. Inactivating antibodies of IL-7 and TSLP that are currently in clinical development may now have another use in the treatment of leukemia and need to be studied in this context. Investigations using these antibodies along with JAK inhibitors are under way. This work has the potential to identify novel therapeutic targets for inhibition, in combination with MTIs for the treatment of ALL, and point to growth factor–mediated signaling as one mechanism of MTI resistance.

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