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IL-7 Activates the Phosphatidylinositol 3-Kinase/AKT Pathway in Normal Human Thymocytes but Not Normal Human B Cell Precursors[1]

Sonja E. Johnson,* Nisha Shah,* Anna A. Bajer,* and Tucker W. LeBien[2]*†

IL-7 signaling culminates in different biological outcomes in distinct lymphoid populations, but knowledge of the biochemical signaling pathways in normal lymphoid populations is incomplete. We analyzed CD127/IL-7Rα expression and function in normal (nontransformed) human thymocytes, and human CD19$^+$ B-lineage cells purified from xenogeneic cord blood stem cell/MS-5 murine stromal cell cultures, to further clarify the role of IL-7 in human B cell development. IL-7 stimulation of CD34$^+$ immature thymocytes led to phosphorylation (p-) of STAT5, ERK1/2, AKT, and glycogen synthase kinase-3 β, and increased AKT enzymatic activity. In contrast, IL-7 stimulation of CD34$^-$ thymocytes (that included CD4$^+$/CD8$^+$ double-positive, and CD4$^+$ and CD8$^+$ single-positive cells) only induced p-STAT5. IL-7 stimulation of CD19$^+$ cells led to robust induction of p-STAT5, but minimal induction of p-ERK1/2 and p-glycogen synthase kinase-3 β. However, CD19$^+$ cells expressed endogenous p-ERK1/2, and when rested for several hours following removal from MS-5 underwent de-phosphorylation of ERK1/2. IL-7 stimulation of rested CD19$^+$ cells resulted in robust induction of p-ERK1/2, but no induction of AKT enzymatic activity. The use of a specific JAK3 antagonist demonstrated that all IL-7 signaling pathways in CD34$^+$ thymocytes and CD19$^+$ B-lineage cells were JAK3-dependent. We conclude that human CD34$^+$ thymocytes and CD19$^+$ B-lineage cells exhibit similarities in activation of STAT5 and ERK1/2, but differences in activation of the PI3K/AKT pathway. The different induction of PI3K/AKT may at least partially explain the different requirements for IL-7 during human T and B cell development. *The Journal of Immunology,* 2008, 180: 8109–8117.

The role of IL-7, and its receptor subunits the IL-7Rα-chain (CD127) and the γ common (γc)[3] chain (CD132), have been extensively studied in murine lymphopoiesis (1, 2). Following its original description as a B-lineage stimulatory molecule (3), the importance of IL-7 was clarified by the demonstration that targeted deletion of the IL-7 (4) or CD127/IL-7Rα-chain (5) genes led to profound disruption of T and B cell development in adult mice. Targeted deletion of the genes encoding the γc subunit of the receptors for IL-2, 4, 7, 9, 15, and 21 (6, 7), and the JAK3 tyrosine kinase (8, 9), also led to severe disruptions of both lineages, consistent with the known structure of the IL-7R and the activation of JAK3 following IL-7 stimulation.

Studies from a number of laboratories some years ago (reviewed in Ref. 10) revealed that human CD19$^+$/surface μ$^-$ B cell precursors (i.e., cells not yet expressing the BCR) express CD127, and

when stimulated with exogenous IL-7 display modestly increased survival. Our understanding of the role of IL-7 in human B cell development has also been aided by analysis of patients with congenital immunodeficiency. SCID patients with mutations in genes encoding components of the IL-7R signaling pathway have frequently been cited as evidence that human B cell development is IL-7 independent. X-linked SCID patients with *IL2RG* mutations have severe defects in T and NK cell development, but have normal or even elevated numbers of peripheral blood B cells (11). Similarly, patients with autosomal recessive mutations in the JAK3 tyrosine kinase exhibit a lymphocyte developmental phenotype indistinguishable from X-linked SCID (T$^-$/NK$^-$/B$^+$) (12, 13). An initial report describing two patients with T$^-$/NK$^+$/B$^+$ non-X-linked SCID attributed this phenotype to mutations in CD127/IL-7Rα, and these patients also had normal/elevated numbers of peripheral blood B cells (14). Two more recent reviews have indicated that mutations in CD127/IL-7Rα are the third most common cause of SCID (15, 16), and normal or elevated percentages or absolute numbers of peripheral blood B cells were present in essentially all these patients. Thus, there is no doubt that circulating B cells in these collective SCID patients do not have an absolute requirement for IL-7 in their development. However, caution should be exercised in extrapolating normal peripheral blood B cell numbers in these SCID patients to the notion that B cell development throughout human life is IL-7 independent. To date, there is no published data on the characteristics of marrow B cell development (i.e., Ig gene rearrangement, V gene usage, pre-BCR expression, cell cycle status, etc.), or the origin (i.e., B-1 vs B-2) and functional capability of circulating B cells, in SCID patients with CD127/IL-7Rα mutations. The requirement for IL-7 signaling could also vary in humans as a function of ontogeny, with fetal/neonatal B cell development being relatively IL-7 independent as occurs in the mouse (17–22). In a potential analogy, although we reported the IL-7-independent development of human

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[3] Abbreviations used in this paper: γc, γ common chain; BM, bone marrow; p, phosphorylated; SP, single-positive thymocyte; DP, double-positive thymocyte; GSK3β, glycogen synthase kinase-3 β; sμHC, surface μ H chain; PTEN, phosphatase and tensin homologue; SOCS-1, suppressor of cytokine signaling-1.

B-lineage cells (23), it is noteworthy that our previous study used 20–22-wk-gestation human fetal bone marrow (BM) as a source of stem cells. It is, therefore, possible that IL-7 signaling differs in fetal, pediatric, and adult human B cell development.

IL-7-induced dimerization of the CD127/IL-7Rα and γc subunits leads to a complex pattern of biochemical signaling that varies with lymphocyte developmental stage and activation status (1, 2, 24). Prominent pathways involve JAK3/STAT5, PI3K/AKT, some Bcl-2 family members, and to a lesser extent RAS/MAPK and *src* family kinases. Regulation of IL-7 signaling during murine thymocyte development is particularly complex, reflecting changes in CD127/IL-7Rα expression during the course of thymocyte development (25), and modulation of signaling by negative regulators such as phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and suppressor of cytokine signaling-1 (SOCS-1) (26, 27). The expression of the IL-7R and functional consequences (e.g., proliferation) of IL-7 stimulation in human thymocytes (28–31) and human fetal B cell precursors (32) have been reported. However, a side-by-side comparison of CD127/IL-7Rα (hereafter referred to as CD127) expression and IL-7 signaling pathway activation in normal human thymocytes and normal human B cell precursors has not been reported. We reasoned that such a study may reveal differences in IL-7 signaling in human T and B cell precursors, which in turn may help explain the lymphocyte developmental status in SCID patients with mutations in the IL-7R signaling pathway. The current study describes the results of this undertaking.

Materials and Methods

Origin and isolation of cells

The acquisition/processing of human cord blood, and the establishment and characterization of the human cord blood CD34$^+$ stem cell/murine MS-5 stromal cell xenogeneic culture for studying human B cell development, has been described in detail (33). CD19$^+$ cells used for analysis of IL-7-mediated survival and signaling in the current study were isolated from 4-wk CD34$^+$/MS-5 cultures by positive selection using anti-human CD19 microbeads (Miltenyi Biotec), as described (33). As we have reported, these cells are CD10$^+$/CD19$^+$/CD22$^+$/CD24$^+$/CD20$^{+/-}$ and <5% express sμHC (see Fig. 1 in Ref. 33).

Thymic tissue was obtained from pediatric patients undergoing cardiac surgery, and was acquired by staff from the Tissue Procurement Facility, University of Minnesota Cancer Center. Thymic tissue was teased into a single-cell suspension in MEM/10% FBS (Invitrogen) and total thymocytes were separated from contaminating erythrocytes on a Ficoll-Hypaque density gradient (Sigma-Aldrich). Total thymocytes were further fractionated into CD34$^+$ and CD34$^-$ subpopulations using Magnetic Affinity Cell Sorting CD34-microbeads (Miltenyi Biotec) according to the manufacturer's instructions, but modified to require only 25% of the recommended Ab concentration. The positively selected CD34$^+$ thymocytes and negatively selected CD34$^-$ thymocytes were resuspended in MEM/10% FBS. The CD34$^+$ thymocyte subpopulation was 95–97% CD34$^+$ upon staining with anti-CD34-PE (clone 8G12; BD Biosciences) and analysis by flow cytometry.

Human fetal and adult BM Ficoll-Hypaque interface cells were thawed from liquid nitrogen storage, washed 3× with FACS buffer (1× PBS, 2.5% FBS, and 0.02% sodium azide), and stained with CD34-PerCP (clone 8G12), CD19-allophycocyanin (clone 25C1), and CD127-biotin (clone eBioRDR5; eBiosciences), detected with streptavidin-PE (Invitrogen). Approval for use of cord blood, fetal and adult BM, and thymic tissue was obtained from the Institutional Review Board, University of Minnesota.

Flow cytometry and cell sorting

Thymocytes were stained with the fluorochrome-labeled mAb CD34-FITC (clone 8G12), CD8-FITC, CD8-PE (clone SK1), and CD4-allophycocyanin (clone SK3) all from BD Biosciences. CD127-PE (clone R34.34) was purchased from Immunotech. Fluorescent staining was evaluated on a FACSCalibur (BD Biosciences) and the acquired data was analyzed using FloJo software (TreeStar). CD34$^-$ thymocytes isolated following magnetic bead depletion of CD34$^+$ cells were further sorted into CD4$^+$/CD8$^+$ dou-
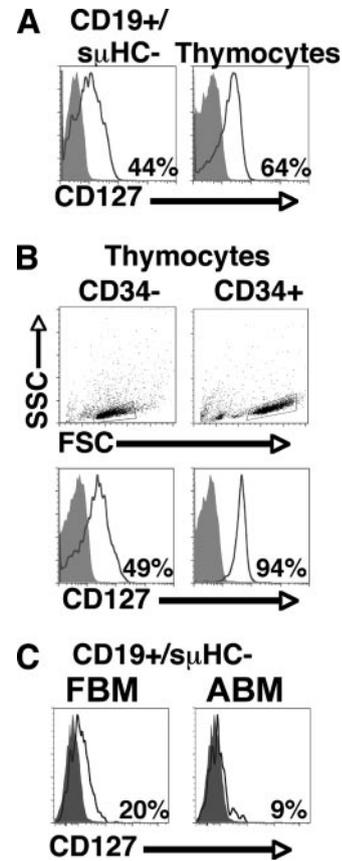

FIGURE 1. CD127 expression on human CD19$^+$ B-lineage cells and thymocytes. CD127 expression was quantified on total thymocytes, and CD19$^+$ cells isolated from a xenogeneic culture (*A*), CD34$^+$ and CD34$^-$ thymocytes (*B*), and cryopreserved fetal or adult BM CD19$^+$/sμHC$^-$ cells (*C*) by staining with PE anti-CD127 or biotinylated anti-CD127 plus PE-streptavidin. Cells were purified as described in the *Materials and Methods*. Solid histograms represent isotype staining controls. Numerical values in the lower corner of histograms represent percentage of CD127$^+$ cells, and are representative of greater than 5 thymic donors, 10 xenogeneic cultures, and 3 samples each of fetal or adult cryopreserved BM. Note the larger cell size (i.e., higher forward light scatter) in CD34$^+$ compared with CD34$^-$ thymocytes.

ble-positive (DP), CD4$^+$/CD8$^-$ single-positive (SP), and CD8$^+$/CD4$^-$ SP using a FACSAria (BD Biosciences).

Short-term stimulation and culture

Lymphoid populations were typically suspended to a concentration of 2–5 × 10^6/ml in MEM/10% FBS in short-term (1–30 min at 37°C) IL-7 stimulation assays. Purified CD34$^+$ thymocytes were cultured in MEM/10% FBS in 96-well round-bottom microtiter plates at 2–5 × 10^4 cells/well in a final volume of 200 μl. CD19$^+$ cells were treated in the same manner, but were plated in the absence or presence of confluent MS-5 stromal cells. Recombinant human IL-7 (obtained as a gift from the Biopharmaceutical Development Program, National Cancer Institute-Frederick, Frederick, MD) was added at 10 ng/ml. Cell numbers were quantified using a microsphere flow cytometric assay (32).

Signaling pathway inhibitors

The PI3K inhibitor LY294002 (34) and the MEK inhibitor U0126 (35) were purchased from EMD Biosciences. The latter was used to probe the role of the ERK1/2 pathway in IL-7 signaling by virtue of the phosphorylation of ERK1/2 by MEK (35). The highly specific JAK3 antagonist PF-956980 was a kind gift from Donnie W. Owens, Pfizer Global Research and Development (Groton, CT). PF-956980 is an analog of the parent drug CP-690,550 (36) that is currently being used in clinical trials for treatment of transplant rejection, rheumatoid arthritis, and psoriasis. All inhibitors

were dissolved in DMSO and used at the concentrations indicated in the figure legends.

Western blotting

Approximately $1–2 \times 10^6$ thymocytes, or CD19$^+$ cells from xenogeneic cord blood CD34$^+$ cell/MS-5 cultures, were stimulated at 37°C with log range concentrations of IL-7 for varying times to evaluate signaling pathway activation. In some experiments, cells were preincubated with inhibitors for 2 h and then stimulated with IL-7. Following stimulation, cells were washed with PBS and lysed in RIPA buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 22 μg/ml aprotinin, 0.01 μM iodoacetamide, 1 μg/ml leupeptin, 0.5 mM sodium fluoride, and 20 μM sodium orthovanadate). The protease inhibitors were obtained from Sigma-Aldrich. Between 10 and 20 μg of protein was electrophoresed per lane on a 4–12% gradient SDS-PAGE gel (Invitrogen). Following electrophoresis, the proteins were transferred to nitrocellulose membranes, incubated for 6 h in 5% milk or 5% BSA dissolved in 1× TBST (50 mM Tris, 150 mM NaCl, and 0.1% Tween 20), and sequentially probed with individual Abs. Blots were incubated overnight (either at room temperature or 4°C) with primary Abs in 1× TBST/5% BSA, 1× TBST/1% BSA, or 1× TBST/5% milk. They were then washed three times (5 min/wash) with 1× TBST, and probed with either sheep anti-mouse HRP (1/5000) or donkey anti-rabbit HRP (1/2500), both from Amersham Life Science in 1× TBST/5% milk for 4 h. Incubations and washes were conducted at room temperature. Following three 5 min washes in 1× TBST, the blots were developed using ECL (Pierce). Primary Abs used for blotting included anti-phosphorylated (p) glycogen synthase kinase-3 β (GSK3β) used at 1/500 (catalog no. 9336), anti-p-AKT used at 1/250 (catalog no. 9271), and anti-AKT used at 1/500 (catalog no. 9272) all from Cell Signaling Technology. Anti-p-ERK1/2 (catalog no. SC-7383; Santa Cruz Biotechnology) was used at 1/100, anti-p-STAT5 (catalog no. 05–495; Upstate Cell Signaling Solutions) was used at 1/500, and anti-human β-tubulin (E7 hybridoma tissue culture supernatant) was used neat.

Chemiluminescent images were scanned using a Bio-Rad Gel Doc GS700 and analyzed using Molecular Analyst software (Bio-Rad).

AKT enzymatic assay

Detection of AKT enzymatic activity was conducted using a nonradioactive AKT kinase assay kit (catalog no.9840; Cell Signaling Technology). In brief, $2–5 \times 10^6$ cells were disrupted in 300 μl of cell lysis buffer. Lysates were vortexed, incubated on ice for 15 min, and microcentrifuged for 10 min at $20,000 \times g$. Supernatants were separated from pelleted debris and either assayed immediately or stored at −80°C and assayed 1–2 wk later. Immunoprecipitation was performed by adding 20 μl of immobilized anti-AKT beads to 300 μl of cell lysate and rocking the mixture overnight at 4°C. The beads were then pelleted, and washed twice in 500 μl of cell lysis buffer and twice in 500 μl of kinase buffer. Pellets were resuspended in 25 μl of 1× kinase buffer, 0.5 μl of 10 mM ATP, and 1 μl of GSK3β fusion protein. The beads were incubated for 30 min at 30°C, and the kinase reaction was terminated by adding 12 μl of sample buffer and 3 μl of 2-ME. The beads were boiled for 5 min, pelleted by microcentrifugation, and the supernatants Western blotted.

Results

CD127 expression on thymocytes and B-lineage cells

Before characterizing IL-7-induced signaling pathways in normal human thymocytes and B cell precursors, we analyzed the expression of CD127 by flow cytometry. Total human thymocytes exhibited heterogeneous expression of CD127 (Fig. 1A). We used CD34 microbeads to purify CD34$^+$ and CD34$^-$ cells and confirmed that CD34 was expressed on 1–2% of total thymocytes (data not shown). Fig. 1B shows that CD127 was expressed on >90% of CD34$^+$ thymocytes and ~50% of CD34$^-$ thymocytes, consistent with previous studies (29–31). As we have reported (33), CD19$^+$ cells purified from xenogeneic CD34$^+$ cord blood stem cell/MS-5 murine stromal cell cultures had heterogeneous expression of CD127, similar to total thymocytes (Fig. 1A). For comparison, CD127 expression on cryopreserved CD34$^+$/CD19$^+$/sμHC cells from fetal and adult BM was also assessed. In general agreement with previously published reports (32, 37), CD127 expression was detected on ~20% of CD34$^+$/CD19$^+$/sμHC$^-$ cells from fetal BM, but only on a minor subfraction (<10%) of
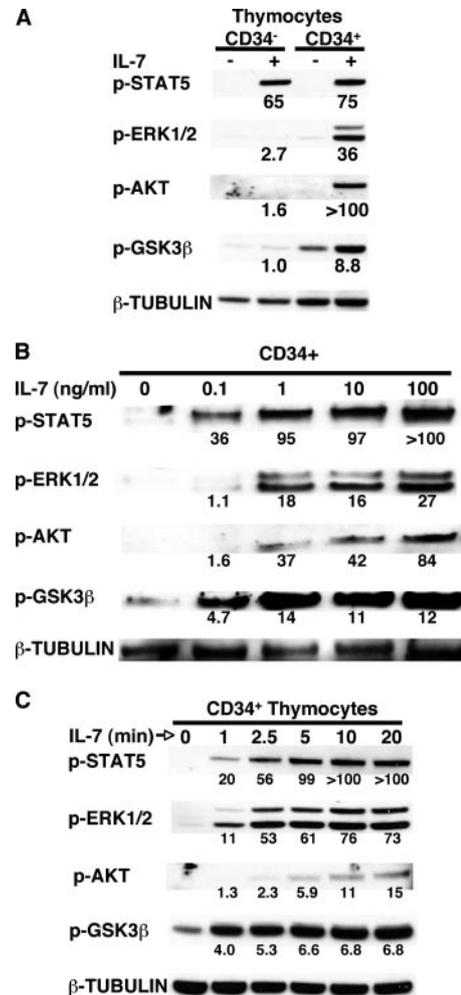

FIGURE 2. IL-7-induced signaling varies between CD34$^+$ and CD34$^-$ thymocytes. *A*, CD34$^+$ and CD34$^-$ thymocytes were stimulated without (−) or with (+) 10 ng/ml IL-7 for 20 min. *B*, CD34$^+$ thymocytes were stimulated with IL-7 at the indicated concentrations for 20 min. *C*, CD34$^+$ thymocytes were stimulated with 10 ng/ml IL-7 for the indicated times. The cells were then lysed, electrophoresed by SDS-PAGE, and sequentially Western blotted with Abs to specific phosphoproteins (p-). Chemiluminescent images were quantified by scanning densitometry. The numerical values represent fold-increase in expression following stimulation with IL-7 compared with no IL-7, and normalized to β-tubulin as a loading control.

CD34$^+$/CD19$^+$/sμHC$^-$ cells from adult BM (Fig. 1C). We were unable to evaluate CD127 expression on CD34$^+$/CD19$^+$/sμHC$^-$ cells from fresh cord blood due to the paucity of pro-B cells.

IL-7 signaling in thymocytes

We first evaluated the IL-7 signaling response in CD34$^+$ and CD34$^-$ thymocytes by Western blotting using Abs to phosphorylated signaling molecules. IL-7 induced p-STAT5 in both CD34$^+$ and CD34$^-$ thymocytes (Fig. 2A), but only CD34$^+$ thymocytes exhibited IL-7-dependent induction of p-ERK1/2, p-GSK3β, and p-AKT. The induction of p-AKT was detected with an Ab to phosphorylated serine 473 that has been widely used as a surrogate for AKT activation subsequent to PI3K activation (38). The Ab used to detect p-GSK3β recognizes phosphorylated serine 9, the primary amino acid phosphorylated by activated AKT (39). As shown in Fig. 2B, IL-7 induced a concentration-dependent appearance of p-STAT5, p-ERK1/2, p-AKT, and p-GSK3β in CD34$^+$ thymocytes. Maximal induction was achieved at 1 ng/ml. Fig. 2C shows
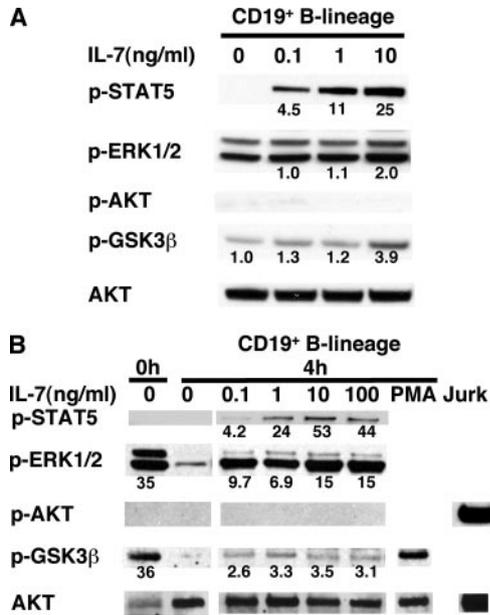
FIGURE 3. CD19^{+} B-lineage cells isolated from xenogeneic cultures are responsive to IL-7 stimulation. *A*, CD19^{+} cells were isolated from a xenogeneic culture, stimulated with IL-7 at the indicated concentrations for 15 min, and Western blotted. *B*, CD19^{+} cells were isolated from a xenogeneic culture and a fraction were lysed immediately (0 h). The remainder were rested for 4 h in MEM/10% FBS, stimulated with the indicated concentrations of IL-7 or 4 nM PMA for 15 min, and Western blotted. Jurkat cell lysate was used as positive control for detection of p-AKT, since the PI3K/AKT pathway is constitutively activated in the cell line due to absence of the lipid phosphatase PTEN. Scanning densitometry values represent fold-increase in expression compared with cells cultured for 4 h and not stimulated with IL-7 (*second lane*, assigned a value of 1.0), and normalized to total AKT as a loading control. The PMA lane was originally the third lane from the left on the gel; using Adobe Photoshop this lane was cut from its original position and moved to a new location between 100 ng/ml IL-7 and Jurkat cells.

the near simultaneous induction of p-STAT5, p-ERK1/2, and p-GSK3β 1 min following IL-7 stimulation of CD34^{+} thymocytes, with maximal induction occurring between 5 and 10 min. The induction of p-AKT appeared to have slightly delayed kinetics, but was likely attributable to differences in the sensitivity of the individual Abs in Western blotting. Three separate thymocyte donors were used in Fig. 2 validating the reproducibility of IL-7 signaling pathway activation in these cells. Although the data in Fig. 2*A* showed no IL-7 induction of p-ERK1/2, p-AKT, or p-GSK3β in CD34^{−} thymocytes, we were interested in whether this was a characteristic common to the major subpopulations. We therefore sorted CD34^{−} cells into CD4^{+}/CD8^{+} DP thymocytes, CD4^{+}/CD8^{−} SP thymocytes, and CD4^{−}/CD8^{+} SP thymocytes. The FACS purified populations were then stimulated with IL-7 and examined in Western blotting. IL-7 induced p-STAT5 in SP thymocytes, and to a much lesser degree in DP thymocytes, but there was little if any detectable p-AKT or p-ERK1/2 induced in any of the three subpopulations (data not shown).

IL-7 signaling in B-lineage cells

We next analyzed IL-7 signaling in CD19^{+} B-lineage cells purified from xenogeneic cultures. Concentration-dependent IL-7 induction of p-STAT5 was readily detected in CD19^{+} B-lineage cells (Fig. 3*A*). In contrast to CD34^{+} thymocytes (Fig. 2), there was weak induction of p-ERK1/2 and p-GSK3β, and induction only occurred at 10 ng/ml IL-7 (Fig. 3*A*). There was no induction

of p-AKT. The inability to detect p-AKT was not a consequence of limiting amounts of total AKT protein in CD19^{+} cells, since easily detectable amounts were present in each lane. CD19^{+} cells lysed immediately following isolation from xenogeneic cultures harbored readily detectable p-ERK1/2, and to a lesser degree p-GSK3β (no IL-7 stimulation lane in Fig. 3*A*). In other experiments, p-AKT was faintly detected in freshly isolated CD19^{+} cells (data not shown), but this was not a consistent observation. We then evaluated the stability of p-ERK1/2 and p-GSK3β by culturing CD19^{+} cells in medium alone for 4 h following removal from MS-5. We refer to these cells as "rested" CD19^{+} cells. Results in Fig. 3*B* show a dramatic reduction of p-ERK1/2 and p-GSK3β in rested CD19^{+} cells. The decrease in p-ERK1/2 was likely due to dephosphorylation of ERK1/2 since total ERK1/2 levels did not change (data not shown). Stimulation of rested CD19^{+} cells with IL-7 led to a robust induction of p-STAT5 and p-ERK1/2. Minimal induction of p-GSK3β was observed. This was not due to a paucity of phosphorylatable GSK3β, as PMA stimulation robustly induced p-GSK3β (Fig. 3*B*). Induction of p-AKT was also not detected in IL-7-stimulated rested CD19^{+} cells. Moreover, this was not attributable to problems with the anti-p-AKT reagent since p-AKT was easily detected in Jurkat lysates (Fig. 3*B*). Similar results to those shown in Fig. 3 were obtained from the analysis of CD19^{+} cells isolated from seven other xenogeneic cultures.

IL-7 induction of AKT enzymatic activity and degradation of p27Kip1

To directly test whether AKT enzymatic activity was enhanced following IL-7 stimulation, we used a nonradioactive enzymatic assay that employs a GSK3β fusion peptide as a substrate. The Jurkat cell line was used as a control. As shown in Fig. 4*A*, IL-7 increased AKT enzymatic activity in CD34^{+} thymocytes, whereas IL-7 did not increase AKT enzymatic activity in CD34^{−} thymocytes (data not shown). These results are consistent with the data in Fig. 2*A* showing the induction of p-AKT in CD34^{+} thymocytes but not CD34^{−} thymocytes. Since rested CD19^{+} cells showed robust induction of p-STAT5 and p-ERK1/2 (Fig. 3*B*), similar to CD34^{+} thymocytes (Fig. 2), we determined whether IL-7 would increase AKT enzymatic activity in rested CD19^{+} cells. As shown in Fig. 4*B*, rested CD19^{+} cells did not undergo increased AKT enzymatic activity following IL-7 stimulation.

IL-7 has been previously shown to induce degradation of the cyclin-dependent kinase inhibitor p27Kip1 in human T cell leukemias and murine IL-7-dependent T cell lines (40, 41), potentially via an unknown ubiquitin E3 ligase (41). IL-7 induced the degradation of p27Kip1 in CD34^{+} thymocytes in a concentration-dependent manner, but had no effect on p27Kip1 levels in CD34^{−} thymocytes (data not shown). We then compared CD34^{+} thymocytes to CD19^{+} cells following incubation in the absence or presence of IL-7 for 1–3 days. IL-7 induced the degradation of p27Kip1 in CD34^{+} thymocytes, but no degradation was detected in IL-7 stimulated CD19^{+} cells (data not shown).

Effect of pathway inhibitors on the IL-7 response

The data in Figs. 2–4 indicated similarities in IL-7-induced activation of p-STAT5 and p-ERK1/2 in CD34^{+} thymocytes and rested CD19^{+} cells, but apparent differences in induction of AKT enzymatic activity and p-GSK3β. To further clarify these differences, we used inhibitors that block pathway activation at three distinct sites: the PI3K inhibitor LY294002 (34), the MEK inhibitor U0126 (35), and the specific JAK3 antagonist PF-956980 (36). We first confirmed the ability of these inhibitors to block components of the IL-7 signaling pathway in a CD127-expressing human pre-B ALL cell line (BLIN-1). As shown in Fig. 5, BLIN-1
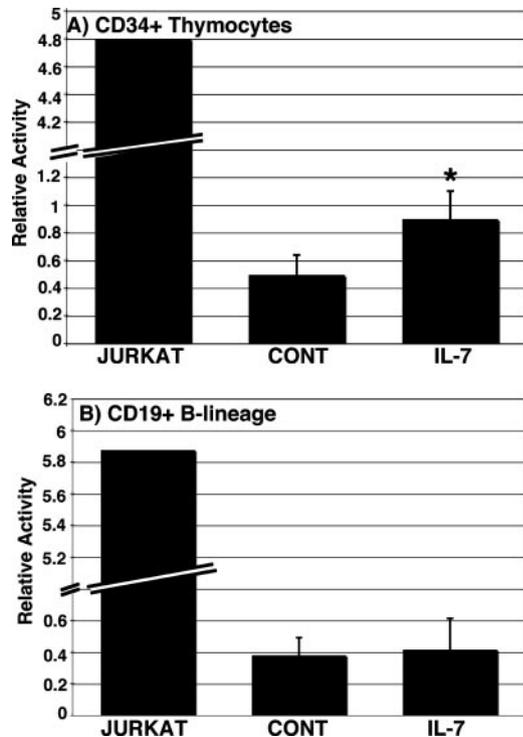
FIGURE 4. IL-7 induction of AKT enzymatic activity occurs in CD34^{+} thymocytes but not CD19^{+} B-lineage cells. CD34^{+} thymocytes (A) and rested CD19^{+} cells (B) were incubated in the absence or presence of 10 ng/ml IL-7 for 20 min. The cells were then tested for AKT enzymatic activity using a nonradioactive AKT kinase kit as described in the *Materials and Methods*. A GSK3β fusion peptide was used as an AKT substrate, and p-GSK3β was detected by Western blotting. The blots were quantified by scanning densitometry and the *y*-axis values represent the relative activity (i.e., AKT enzymatic activity based on degree of GSK3β phosphorylation) normalized to total AKT immunoprecipitated from cell lysates. The *p*-value ($*$) in A is <0.048. Each bar represents the mean $\pm$ SD of three samples.

showed enhanced phosphorylation of STAT5 and AKT in response to IL-7 stimulation. ERK1/2 was constitutively phosphorylated in the absence of IL-7 stimulation. Preincubation of BLIN-1 with LY294002, U0126, or PF-956980 for 2 h before IL-7 stimulation effectively blocked the targeted component of each pathway (Fig. 5). LY294002 blocked p-AKT but not p-STAT5 induction, and had no effect on constitutive p-ERK1/2. U0126 reduced
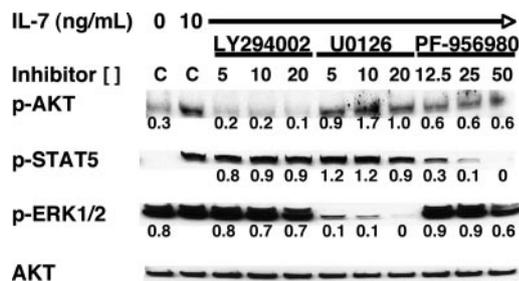

FIGURE 5. Signaling pathway inhibitors selectively block IL-7 induction of phosphoproteins in human pre-B ALL cells. The human pre-B ALL cell line, BLIN-1, was incubated for 2 h in LY294002 (5, 10, or 20 μM), U0126 (5, 10, or 20 μM), JAK3 antagonist PF-956980 (12.5, 25, or 50 nM), or 0.5% vol/vol DMSO as a control (C). Cells were stimulated with IL-7 for 15 min at 37°C and Western blotted. Scanning densitometry values represent fold-change in expression compared with IL-7-stimulated cells that were assigned a value of 1.0, and normalized to total AKT as a loading control.
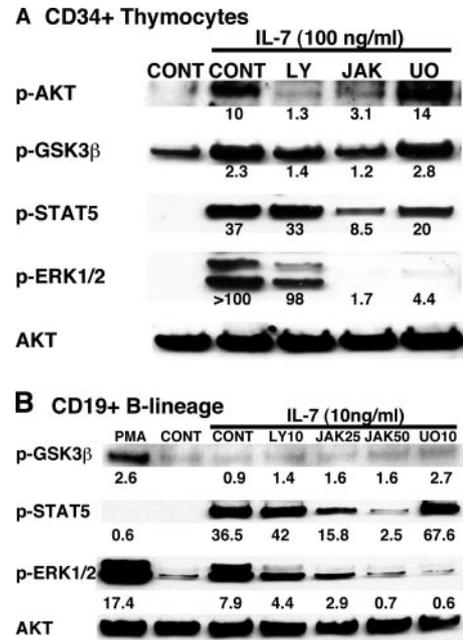

FIGURE 6. Signaling pathway inhibitors selectively block IL-7 induction of phosphoproteins in CD34^{+} thymocytes and CD19^{+} B-lineage cells. CD34^{+} thymocytes (A), and CD19^{+} cells (B) from a xenogeneic culture (that had been rested for 4 h in MEM/10% FBS), were incubated for 2 h in LY294002 (LY, 10 μM), the JAK3 antagonist PF-956980 (JAK, 25 or 50 nM), or U0126 (U0, 10 μM). The first control lane (CONT) represents cells incubated without inhibitors and not stimulated with IL-7. The second control lane represents cells incubated in 0.5% vol/vol DMSO (used to solubilize the inhibitors) for 2 h as a negative control for the effect of the inhibitors. Following 2-h incubation with inhibitors, the cells were washed once, stimulated with IL-7 for 20 min, and Western blotted. Chemiluminescent images were quantified by scanning densitometry. The numerical values represent fold-increase in expression following stimulation with IL-7 compared with no IL-7, and normalized to total AKT as a loading control. PMA (4 nm for 15 min) was used as a positive control for induction of p-GSK3β and p-ERK1/2 in CD19^{+} cells.

constitutive p-ERK1/2, but had no effect on p-STAT5 or p-AKT induction. PF-956980 blocked p-STAT5 induction, reduced p-AKT induction, but had little effect on constitutive p-ERK1/2.

Having confirmed that these inhibitors specifically blocked components of the IL-7 signaling pathway in BLIN-1, we tested their ability to do so in CD34^{+} thymocytes and rested CD19^{+} cells. CD34^{+} thymocytes were preincubated with inhibitors and then stimulated with IL-7. Fig. 6A shows that PF-956980 (JAK) blocked induction of p-STAT5, p-ERK1/2, p-AKT, and p-GSK3β. U0126 blocked induction of p-ERK1/2, but had no effect on p-AKT or p-GSK3β. The potential effect of U0126 on p-STAT5 was not reproducible. LY294002 blocked induction of p-AKT and p-GSK3β, had no effect on p-STAT5, and partially blocked induction of p-ERK1/2. The partial inhibitory effect of LY294002 on p-ERK1/2 suggests that PI3K may potentiate ERK1/2 activation downstream of Ras (see *Discussion*).

Pathway activation in rested CD19^{+} cells stimulated with IL-7 was examined using the same inhibitor protocol. As shown in Fig. 6B, PF-956980 blocked induction of p-STAT5 and p-ERK1/2, whereas U0126 only blocked p-ERK1/2. Similar to CD34^{+} thymocytes, LY294002 partially blocked IL-7 induction of p-ERK1/2 but had no effect on p-STAT5. This experiment shows the complete absence of IL-7 induction of p-GSK3β. However, this could not be explained by an absence of potentially phosphorylatable GSK3β, since PMA treatment led to the appearance of p-GSK3β (Fig. 6B).
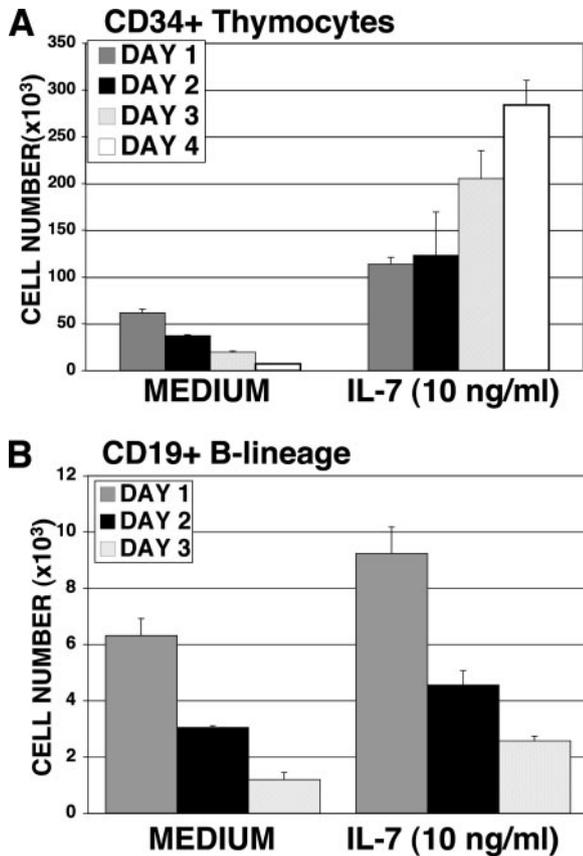
FIGURE 7. IL-7 induces the survival and proliferation of CD34$^+$ thymocytes, but only partial survival in CD19$^+$ B-lineage cells. CD34$^+$ thymocytes (*A*) and CD19$^+$ B-lineage cells isolated from a xenogeneic culture (*B*) were cultured in MEM/10% FBS and cell numbers quantified on the days indicated. Each bar represents the mean of triplicate values ± SD. Note that the y-axis scale is different for the two cell types. Day 0 input cell number for CD34$^+$ thymocytes was $67.9 \times 10^3 \pm 6.0 \times 10^3$, and for CD19$^+$ B-lineage cells was $25.6 \times 10^3 \pm 1.4 \times 10^3$.

IL-7 effects on survival and proliferation

Since IL-7 signaling differed between CD34$^+$ thymocytes and CD19$^+$ B-lineage cells in AKT activation and degradation of p27Kip1, we compared the effect of IL-7 on the survival/proliferation of the two populations. CD34$^+$ thymocytes showed a gradual loss of viable cells when maintained up to 4 days in medium alone (Fig. 7*A*). Inclusion of IL-7 induced a time-dependent increase in proliferation between days 1–4. In contrast, 75–90% of CD19$^+$ cells were dead after 1–2 days in medium alone; IL-7 significantly enhanced survival but failed to induce proliferation (Fig. 7*B*). We also evaluated the effects of LY294002 and U0126 on IL-7 stimulated CD34$^+$ thymocyte proliferation. After 4 days of culture with LY294002, proliferation in response to IL-7 was completely blocked, while no inhibition occurred in the presence of UO126 (data not shown). Assessing the effect of LY294002 and U0126 on IL-7 induced proliferation in CD19$^+$ cells was technically not feasible. CD19$^+$ cells from xenogeneic cultures require the presence of MS-5 to survive. Since the inhibitors must be present for the duration of the assay, the direct effects on proliferation of CD19$^+$ cells cannot be separated from indirect effects on MS-5 stroma.

Discussion

Although considerable published data exists on IL-7 signaling pathways activated in murine and human lymphoid cell lines (1, 2, 24, 25), our understanding of these pathways activated in normal lymphoid progenitors is less complete. The current study was undertaken to elucidate IL-7 signaling pathways activated in normal (nontransformed) immature human thymocytes and B-lineage cells. We reasoned that such an analysis could potentially identify differences in IL-7 signaling in CD34$^+$ thymocytes and CD19$^+$ cells, thereby helping to explain the apparent differences in lineage requirement for this cytokine in human lymphocyte development.

CD34$^+$ thymocytes represent the most immature population in the human thymus (42–44) and respond to IL-7 by undergoing proliferation (29, 30, 45). We, therefore, chose this population for characterizing IL-7 signaling in early human thymocyte development, although we are aware that not all CD34$^+$ thymocytes are T-lineage committed (43). CD19$^+$ cells isolated from cord blood CD34$^+$ stem cell/MS-5 xenogeneic cultures were studied for two reasons. First, we have previously shown that CD19$^+$/CD127$^+$ cells are responsive to IL-7 stimulation based upon flow cytometric analysis of p-STAT5 induction, and can undergo IL-7-induced proliferation when plated on MS-5 (33). Second, the xenogeneic culture provided us with sufficient CD19$^+$ cells to conduct biochemical experiments. CD19$^+$/sμHC$^-$ cells are essentially undetectable in cord blood, and it would have been impractical to repeatedly conduct large scale FACS purification of CD19$^+$ cells from pediatric or adult BM to conduct extensive biochemical studies.

The current study is the first report evaluating IL-7-induced signaling pathways in nontransformed human B cell precursors. Our collective results reveal differences in PI3K/AKT activation between CD34$^+$ thymocytes and CD19$^+$ B cell precursors based on four observations. First, the anti-p-AKT Ab was a useful surrogate marker for detecting AKT activation in IL-7-stimulated CD34$^+$ thymocytes (Figs. 2 and 6), but we could not demonstrate AKT activation using this Ab following IL-7 stimulation of freshly isolated or rested CD19$^+$ cells (Figs. 3 and 6). Second, IL-7 induced AKT enzymatic activity in CD34$^+$ thymocytes (Fig. 4*A*) but not rested CD19$^+$ cells (Fig. 4*B*). Third, IL-7 readily induced phosphorylation of serine 9 on GSK3β (the site phosphorylated by AKT) in CD34$^+$ thymocytes (Figs. 2 and 6*A*), but there was weak to no induction in freshly isolated and rested CD19$^+$ cells (Figs. 3 and 6*B*). Fourth, IL-7 stimulation of CD34$^+$ thymocytes led to proliferation (Fig. 7*A*), and proliferation was completely blocked by the PI3K inhibitor LY294002 (data not shown). In contrast, IL-7 did not induce proliferation in CD19$^+$ cells (Fig. 7*B*).

AKT activation promotes increased cell size, cell division, suppression of apoptosis, inactivation of cell cycle inhibitors, and induction of cyclin and cytokine gene expression (39). By short-term enzymatic and Western blot assays (measured minutes following addition of IL-7), IL-7 has been shown to induce PI3K/AKT activation in T cell ALL (46) and normal human thymocytes (28, 47). However, studies with human peripheral blood T cells (48) and murine splenic/lymph node T cells (49) indicated that IL-7-induced activation of PI3K/AKT has more delayed kinetics, with 3–6 h required for initial detection. Furthermore, naive and memory human CD4$^+$ peripheral blood T cells require 1–2 wk of constant IL-7 stimulation to undergo significant cell cycle entry (50), and human cord blood CD4$^+$ recent thymic emigrants require at least 5 days for manifestation of prominent IL-7-induced cell cycle entry (51). Thus, IL-7 transduces a survival/proliferative signal in several populations of normal and leukemic human T-lineage cells, the kinetics of which vary with the cell type. In contrast, CD34$^-$ thymocytes (of which >70% are DP thymocytes) did not undergo IL-7-induced PI3K/AKT activation (Fig. 2*A*) or proliferation (data not shown). These results are consistent with the widely accepted model wherein the majority of DP thymocytes are destined to undergo apoptosis during thymic selection, and an IL-7-mediated

survival/proliferation signal would need to be attenuated to preclude survival of autoreactive T cells. Murine preselection DP thymocytes fail to respond to IL-7 signaling because the response is inhibited by SOCS-1 (27). It is not known whether this mechanism is operative in human DP thymocytes.

The low to absent AKT activation and phosphorylation of GSK3β following IL-7 stimulation of nontransformed human CD19^{+} B cell precursors (Figs. 3, 4B, and 6B) suggests minimal activation of the PI3K/AKT pathway is occurring. These results accord with the inability of IL-7 to induce proliferation in CD19^{+} cells, in the absence of MS-5 stromal cells (Fig. 7B). The mechanism underlying the failure of IL-7 to activate the PI3K/AKT pathway is unknown. The concern that this could be explained by the heterogeneous expression of CD127 on CD19^{+} cells (Fig. 1 in Ref. 33) is tempered by the ease with which IL-7 induction of p-STAT5 and p-ERK1/2 was detected (Figs. 3 and 6B). Although several reports have described delayed kinetics in PI3K/AKT activation vis a vis STAT5 activation (48, 49), stimulation of CD19^{+} cells with IL-7 for up to 18 h did not result in detectable induction of p-AKT (data not shown). The p85 subunit of PI3K binds to phosphorylated tyrosine 449 on CD127 (52), and a tyrosine > phenylalanine substitution inhibits PI3K-dependent proliferation of IL-7-stimulated murine B-lineage cells (53). Additional evidence supporting a role for PI3K is the impairment of B cell development in mice with a targeted disruption of p85α (54). Given the robust induction of p-STAT5 in IL-7-stimulated CD19^{+} cells (Figs. 3, A and B, and 6B), and the fact that JAK3 phosphorylation of tyrosine 449 on CD127 provides a docking site for STAT5 (24), we assume that phosphorylated tyrosine 449 would also be available for docking of p85 and subsequent activation of PI3K in human CD19^{+} B cell precursors. However, there could be a difference in stoichiometric access to phosphorylated tyrosine 449 that favors binding of STAT5 over p85 in human CD19^{+} cells. Alternatively, additional mechanisms downstream of PI3K activation and PIP3 production may attenuate AKT activation in CD19^{+} cells.

Analysis of p27Kip1 expression following IL-7 stimulation yielded results that paralleled PI3K/AKT activation. IL-7 induced the degradation of p27Kip1 in CD34^{+} thymocytes, but not CD34^{−} thymocytes or CD19^{+} cells (data not shown). AKT phosphorylates p27Kip1 on threonine 157, leading to sequestration by 14-3-3 and exclusion from the nucleus in many cell types (39). Thus, PI3K/AKT activation and AKT-mediated phosphorylation of p27Kip1 is a component of the IL-7 signaling pathway in CD34^{+} thymocytes, which is undetectable in CD34^{−} thymocytes and CD19^{+} cells.

Although activation of ERK1/2 is not a consequence of IL-7 signaling in many lymphoid cells (24), ERK1/2 activation has been reported to at least partially mediate cooperative signaling between the IL-7R and the pre-BCR (55). IL-7 stimulation led to robust induction of p-ERK1/2 in CD34^{+} thymocytes but not CD34^{−} thymocytes (Fig. 2A). As would be expected, the MEK inhibitor U0126 completely blocked IL-7 induction of p-ERK1/2 (Fig. 6A). However, CD34^{+} thymocytes stimulated with IL-7 and coincubated with U0126 showed no decrease in proliferation compared with cells stimulated with IL-7 alone (data not shown), indicating little or no role for ERK1/2 activation in proliferation of these cells. Similar results were reported in studies evaluating the role of IL-7 induction of p-ERK1/2 in human T cell leukemia (46). CD19^{+} cells isolated from xenogeneic cultures and directly stimulated with IL-7 showed minimal induction of p-ERK1/2 (Fig. 3A). However, when rested in medium and stimulated with IL-7, robust induction of p-ERK1/2 occurred (Fig. 3B), and this was blocked by U0126 (Fig. 6B). The enhanced ability of IL-7 to induce p-ERK1/2 in rested CD19^{+} cells may reflect an increased

pool of nonphosphorylated ERK1/2 that accumulated during the 4-h incubation in medium alone (Fig. 3B). This could represent an active process wherein removal of CD19^{+} cells from their xenogeneic microenvironment leads to induction of a MAPK phosphatase, or the release of a MAPK phosphatase from an inhibitory state (56). A related explanation is that endogenous murine IL-7 (or some other cytokine) synthesized by MS-5 may be activating the ERK1/2 pathway in CD19^{+} cells developing in the xenogeneic microenvironment. The subcellular localization of IL-7-induced p-ERK1/2 (and by extension the identity of the phosphorylated substrates) in CD34^{+} thymocytes and CD19^{+} cells, and their role in the development of both populations, remain to be characterized.

The highly specific PI3K inhibitor LY294002 has been widely used at in vitro concentrations of 10–20 μM to block PI3K enzymatic activity. In addition to the expected inhibition of IL-7-induced p-AKT and p-GSK3β in CD34^{+} thymocytes (Fig. 6A), LY294002 partially blocked IL-7 induction of p-ERK1/2 in CD34^{+} thymocytes (Fig. 6A) and CD19^{+} cells (Fig. 6B). One possible explanation is that PI3K may potentiate ERK1/2 activation at the level of MEK, as previously reported for BCR signaling in murine B cells (57, 58). However, if the effect of LY294002 on IL-7 induction of p-ERK1/2 uses the same mechanism in CD34^{+} thymocytes and CD19^{+} cells, then it would likely be AKT independent given the differences in activation of AKT following IL-7 stimulation in these two cell types.

The JAK3 antagonist PF-956980 was a potent inhibitor of IL-7-induced pathway activation in CD34^{+} thymocytes (Fig. 6A) and rested CD19^{+} cells (Fig. 6B). PF-956980 is an analog of the parent compound CP-690,550 that, when tested against a panel of 30 kinases, was shown to have nanomolar potency only against JAK3 (59). JAK3 is an essential proximal signaling kinase in the IL-7 pathway, underscored by the fact that mice with targeted disruptions of JAK3 and CD127 have similar deficiencies in lymphocyte development (5, 8), as do non-X-linked SCID patients with mutations in JAK3 and CD127 (12–14). JAK3 plays a crucial role in phosphorylating tyrosine residues in CD127 (1, 2, 24, 25). However, the mechanism by which IL-7 activates ERK1/2 (likely via the canonical Ras/Raf/MEK pathway) is not understood at the proximal stages of IL-7 signaling. One possibility is that the Grb2 adapter binds to a phosphorylated tyrosine in the CD127 cytoplasmic domain. Although there is no published evidence to support this, the fact that PF-956980 completely blocked induction of p-ERK1/2 (Fig. 6) is consistent with the JAK3-dependent phosphorylation of a CD127 tyrosine residue (or residues) being essential to the eventual activation of ERK1/2.

We cannot rule out the possibility that the presence of CD19^{+}/sμHC^{−}/CD127^{+} cells in xenogeneic culture are attributable to selection or maintenance of this population by MS-5, that could be unique vis a vis what occurs in vivo. The CD19^{+}/CD127^{+} cells that develop from hematopoietic progenitors in this xenogeneic culture exhibit an immunophenotype and pattern of Ig gene rearrangement consistent with early/pro-B cells. However, a global gene expression profile for these cells has not been reported, so the degree to which they may differ from freshly isolated human pro-B cells (60, 61) is unknown. Human (and murine) lymphoid cells maintained on stromal cells for weeks in vitro may also exhibit prolonged signaling responses that lead to a greater propensity to proliferate, at the expense of differentiation, compared with B-lineage cells in vivo.

In conclusion, the current study represents the first side-by-side comparison of IL-7 signaling in nontransformed human thymocytes and human CD19^{+} B cell precursors. The results reveal both similarities and differences in IL-7 pathway activation. Rapid

phosphorylation of STAT5 and induction of the ERK1/2 pathway is characteristic of both cell types. However, there is a substantial difference in the activation of the PI3K/AKT pathway. The difference suggests a mechanism whereby IL-7 is a non-redundant activator of the PI3K/AKT pathway in early $CD34^+$ thymocytes, but not in $CD19^+$ B cell precursors. Our inability to detect activation of PI3K/AKT in normal human B cell precursors may reflect the requirement for one or more additional stromal cell-derived cytokines to cooperate with IL-7. Our results could also provide insight into the lymphocyte developmental phenotype in SCID patients with mutations in the IL-7R signaling pathway (i.e., T^-, B^+, and NK^+). These patients have a profound deficiency in T cell development including minimally developed thymic architecture (15, 16). The capacity of the PI3K inhibitor LY294002 to completely block IL-7 induced proliferation of $CD34^+$ thymocytes strongly implicates this pathway in the numerical expansion of early thymic progenitors. This interpretation is consistent with an earlier report showing that enforced expression of a dominant negative form of PI3K in human thymocytes blocked proliferation (28). In the case of $CD19^+$ B-lineage cells, the fact that IL-7 alone is insufficient to activate the PI3K/AKT pathway suggests that, at least in the human neonate, an additional cytokine (or cytokines) is required for PI3K/AKT activation. How IL-7-induced activation of the JAK3/STAT5 and ERK1/2 pathways works in concert with, or independent of, this putative PI3K/AKT activating cytokine will require further study.

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Disclosures

The authors have no financial conflict of interest.

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