Common γ chain-signaling cytokines promote proliferation of T-cell acute lymphoblastic leukemia

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A B S T R A C T

Background and Objectives. The identification of signals critical for the pathophysiology of T-cell acute lymphoblastic leukemia (T-ALL) should contribute to the development of novel, more effective therapeutic strategies. Common γ-chain signaling cytokines (γc-cytokines) — interleukins 2, 4, 7, 9 and 15 — differentially regulate T-cell development, survival, proliferation and differentiation. Although studies exist on some individual cytokines, no comprehensive analysis of the effects of the γc-cytokine family on malignant T cells has been reported. Here, we examined the effect of γc-cytokines on T-ALL proliferation.

Design and Methods. Primary leukemic cells were collected at diagnosis from the blood or bone marrow of children with T-ALL. The cells were immunophenotyped and classified according to maturation stage. Proliferative responses to γc-cytokines were assessed by ^{3}H-thymidine incorporation.

Results. All γc-cytokines promoted proliferation of primary T-ALL cells. Interleukin (IL)-7 was the cytokine that most frequently induced leukemic cell proliferation and promoted the most robust responses. IL-4 preferentially stimulated proliferation of samples with a more mature immunophenotype, whereas CD1a-positive cortical T-ALL cells were less responsive to IL-9. Finally, combinations of two γc-cytokines showed synergistic or additive proliferative effects.

Interpretation and Conclusions. This study indicates that all the γc-cytokines tested can stimulate proliferation of leukemic T cells and suggests that synergistic effects may occur *in vivo*. We present the first demonstration that IL-9 and IL-15 can provide a proliferative signal to T-ALL cells. Importantly, our results support the hypothesis that IL-7 may function as a critical regulator of T-ALL and that its activity may be potentiated by other γc-cytokines.

Key words: T-ALL, IL-7, common γ chain, proliferation.

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Increasing evidence suggests that, in addition to mechanisms intrinsic to the tumor cell, exogenous (non-cell autonomous) signals may also contribute to tumor development. Interleukin (IL)-2, IL-4, IL-7, IL-9 and IL-15 belong to a family of cytokines whose receptors share the common γ chain (γc). Despite some redundancy, these cytokines that signal through the γc (γc-cytokines) have essentially distinct and often critical effects in normal T-cell development, survival, proliferation and differentiation. Importantly, they have also been implicated, directly or indirectly, in T-cell leukemogenesis. Numerous studies have demonstrated that IL-7 is not only critical for normal thymocyte development, but might also contribute to the pathogenesis of T-cell leukemia.[1-4] Several studies suggest that IL-2 might have a positive effect on the progression of T-cell leukemia. T-ALL cells express the receptors for IL-2,[4,9-11] secrete functional IL-2,[12] and proliferate in response to the cytokine,[1,10-14] raising the possibility that an IL-2 autocrine loop may exist in some cases of T-ALL. In contrast to IL-2 and IL-7, little evidence has been gathered regarding the involvement of IL-4 in T-ALL. Nonetheless, IL-4 was shown to mediate T-ALL proliferation,[1,14] and survival.[4] To our knowledge, no studies have been published on the effect of IL-9 or IL-15 in T-ALL, although it has been demonstrated that IL-9 is a major *in vitro* anti-apoptotic[15] and growth factor[16] for thymic T-cell lymphomas, and IL-15 transgenic mice develop fatal T-NK lymphocytic leukemia.[17] Here, we show that the γc-signaling cytokines IL-2, IL-4, IL-7, IL-9 and IL-15 induce proliferation of primary T-ALL cells, and may exert synergistic effects on malignant T cells. IL-7 is the γc-cytokine that most frequently and more vigorously promotes a

proliferative response. Interestingly, the effects of IL-7, as well as IL-2 and IL-15, are independent of the T-ALL maturation status. In contrast, responsiveness to IL-4 and IL-9 seems to be related to the developmental stage of T-ALL cells. Overall, our data support the hypothesis that γc-cytokines, particularly IL-7, may play a relevant role in the biology of T-ALL, and that γc-mediated signaling may constitute a valid target for therapeutic intervention.

Design and Methods

Primary T-ALL samples

T-ALL cells were obtained from the peripheral blood and/or the bone marrow of newly diagnosed pediatric patients with high leukemia involvement (85-100%). Informed consent was obtained and the studies were approved by the DFCI Institutional Review Board. Samples were enriched by density centrifugation over Ficoll-Hypaque and washed twice in RPMI-1640 supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 2 μM L-glutamine (henceforth referred to as RPMI-10). T-ALL samples were classified according to the *European Group for Immunological Characterization of Leukemias* (EGIL) criteria, by immunophenotypic analyses using fluorochrome-conjugated monoclonal antibodies for CD1a, CD2, CD3, CD5, CD7 and CD8.

Proliferation assay

Cells were cultured (2×10^6 cells/mL) in triplicate in RPMI-10, in flat-bottomed 96-well plates at 37°C with 5% CO_2. Cultures were carried out for 72 or 96 hours in medium without cytokines (control condition), with a single γc cytokine or with combinations of two of the following cytokines: IL-2 (100 U/mL), IL-4 (10 ng/mL), IL-7 (10 ng/mL), IL-9 (50 U/mL), or IL-15 (20 ng/mL) (purchased from R&D Systems, Minneapolis, MN, USA; Endogen, Woburn, MA, USA and Peprotech, Rocky Hill, NJ, USA). Cells were incubated with ^{3}H-thymidine (1 μCi/well) for 16 hours prior to harvest. DNA synthesis was assessed by ^{3}H-thymidine incorporation, which was measured using a liquid scintillation counter. The proliferation index (PI) was calculated as:

$$PI = \frac{\text{cpm experimental condition}}{\text{cpm control condition}}$$

A cytokine was considered to induce proliferation when the PI was at least 1.5 and the mean of the triplicates was higher than 2 standard deviations of the control.

Statistical analysis

Differences between the proliferative effects of cytokines were assessed using the non-parametric Wilcoxon signed ranks test. Differences between two (i.e. $CD1^+$ versus $CD1^-$ samples) or more (i.e. EGIL classification stages) independent variables (i.e. populations) regarding the degree of proliferation in response to each cytokine or the intensity of expression of a certain marker, were evaluated using 2-tailed non-parametric Mann-Whitney test and non-parametric Kruskal-Wallis test, respectively. Differences were considered statistically significant for $p<0.05$.

Results

All γc-signaling cytokines can induce proliferation of T-ALL cells: IL-7 and IL-4 induce stronger responses

T-ALL cells (n=25) were enriched by density centrifugation, immunophenotyped and classified according to their maturation stage using the criteria defined by the European Group for Immunological Characterization of Leukemias (EGIL).[18] Primary T-ALL samples, all of which expressed mRNA for γc (*data not shown*), were cultured *in vitro* for 72 and/or 96 hours in medium alone or in the presence of IL-2, IL-4, IL-7, IL-9 or IL-15. All the γc-cytokines induced proliferation of T-ALL cells, although a considerable interpatient variation was observed (Figure 1A,B). IL-7 was the cytokine that stimulated responses in the highest number of patients (18 of 25; 72%), followed by IL-4 (64% of the cases). IL-2 and IL-9 induced proliferation in, respectively, 44% and 40% of the patients, whereas IL-15 only stimulated responses in 24% of the patients. Importantly, nearly all patients (23 of 25; 92%) responded to at least one of these cytokines. The majority of the patients (n=16; 64%) showed a proliferative response to at least 2 different cytokines, with IL-7 being one of the stimulatory cytokines in all these cases (Figure 1A,B). None of the patients responded exclusively to IL-2, IL-9 or IL-15. Confirmation that the effects of γc-cytokines were exerted on malignant T-cells was obtained by assessing the T-cell receptor (TCR) clonality of the proliferating cells (using patient-specific primers)[19] or lack of surface CD3 expression, when suitable (*data not shown*). The magnitude of the T-ALL proliferative responses to the γc-cytokines was analyzed subsequently. In most T-ALL samples responsive to multiple cytokines (81%; 13 in 16), including the 3 patients who responded to all cytokines (T-ALL# 2, 5 and 15), IL-7 was the most potent proliferative stimulus (Figure 1A, B). Moreover, one-third of the IL-7-responsive patients (6 of 18) showed robust proliferation, with a PI greater than 10

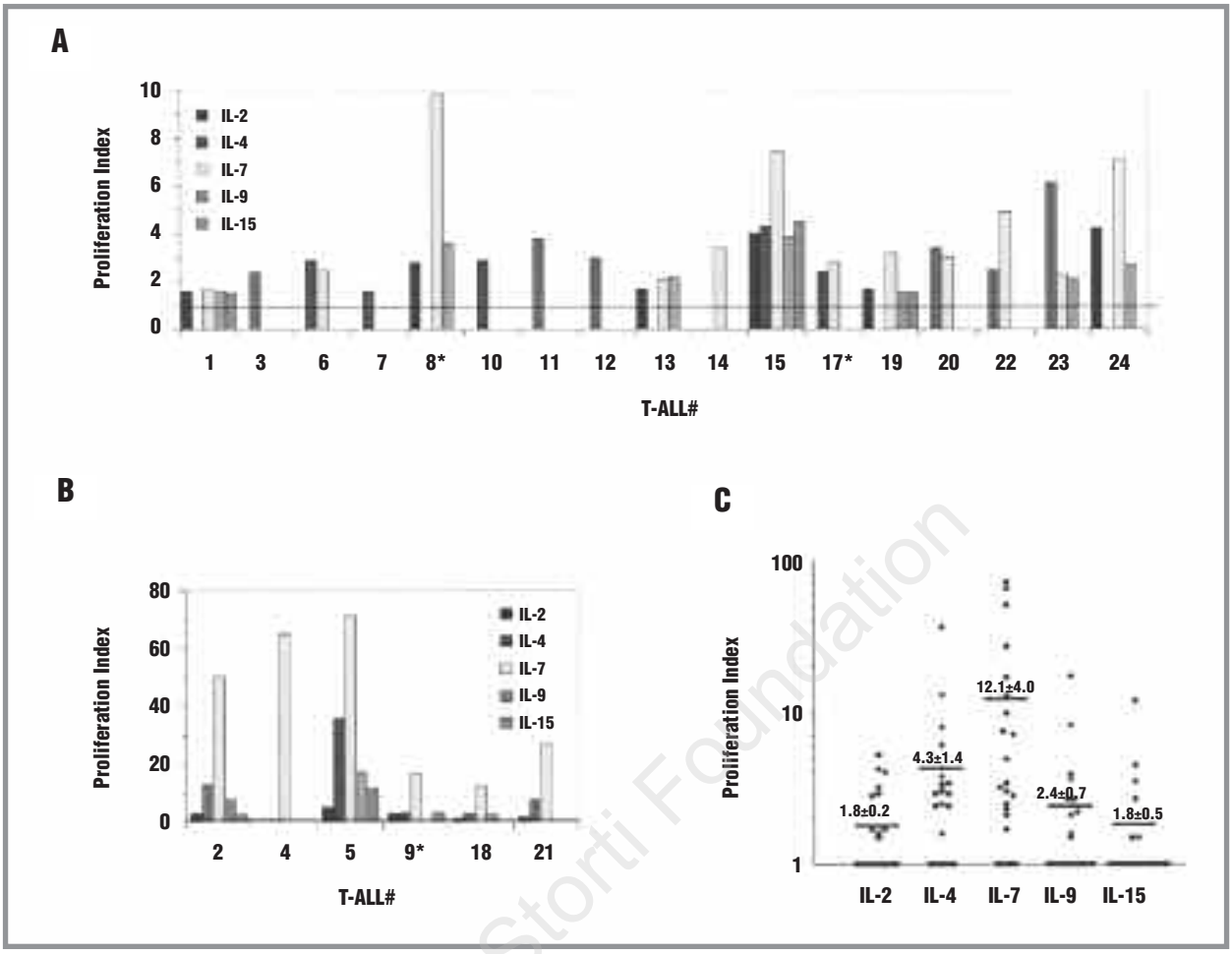

Figure 1. Proliferative responses of primary T-ALL samples to stimulation with γc-signaling cytokines. The proliferation index was calculated for 72 hours of culture, except when indicated (*, 96 hours). Columns represent proliferative responses to cytokines (PI ≥ 1.5) for each patient. Unresponsive cases are not shown (dashed line represents PI=1). No statistically significant inhibitory effects were observed. (A) Samples showing responses to cytokines with PI≤10. (B) Samples with at least one robust proliferative response to cytokine(s) (PI>10). T-ALL samples were classified according to the EGIL criteria.[18] Pre/pro-T-ALL: #1, 8, 13, 19, 22; Cortical T-ALL: #2, 4, 6, 7, 9, 10, 14, 16, 17, 20, 21, 25; Mature T-ALL: #3, 5, 11, 12, 15, 18, 23, 24. (C) T-ALL proliferative responses per individual γc-cytokine. Each symbol corresponds to the PI of one T-ALL specimen in response to the indicated cytokine. Data are presented on a logarithmic scale, with non-responsive cases displayed as having a PI of 1. Mean PI ± SEM are indicated for each cytokine.

(Figure 1B), a frequency considerably higher than that for the other γc cytokines (IL-2, 0 cases in 11; IL-4, 2 cases in 16; IL-9, 1 case in 10; IL-15, 1 case in 6). Thus, IL-7 was not only the cytokine that stimulated a higher percentage of patients but, strikingly, it also induced the strongest proliferative effect in most of the responders. In the other 3 multiple responders the most robust proliferation was induced by IL-4 (T-ALL #6 and #20) or IL-9 (T-ALL #13). Comparison of the average proliferative effects of γc cytokines on T-ALL confirmed that IL-7 induced the most robust response (mean PI±SEM, 12.1±4.0; Figure 1C), which was significantly higher than that induced by IL-4 (4.3±1.4; p=0.018; Wilcoxon's test), IL-9 (2.4 ± 0.7; p<0.001), IL-2 (1.8±0.2; p<0.001) or IL-15 (1.8±0.5; p<0.001). Likewise, responsiveness to IL-4 was significantly higher than that to IL-9 (p=0.018), IL-2 (p=0.011) or IL-15 (p<0.001). Comparison of the proliferative effects of IL-2, IL-9 and IL-15 on T-ALL cells did not show statistically significant differences (p>0.05). In summary, our studies clearly demonstrate that IL-7, and to a lesser degree IL-4, are the cytokines that mediated the most intense and more frequent T-ALL proliferative responses.

IL-7 and IL-4 effects on T-ALL proliferation show contrasting kinetics

Since IL-7 and IL-4 were the γc-cytokines that elicited stronger proliferative responses, we evaluated the progression over time of the proliferative responses of T-ALL to these cytokines (Figure 2). Interestingly, in

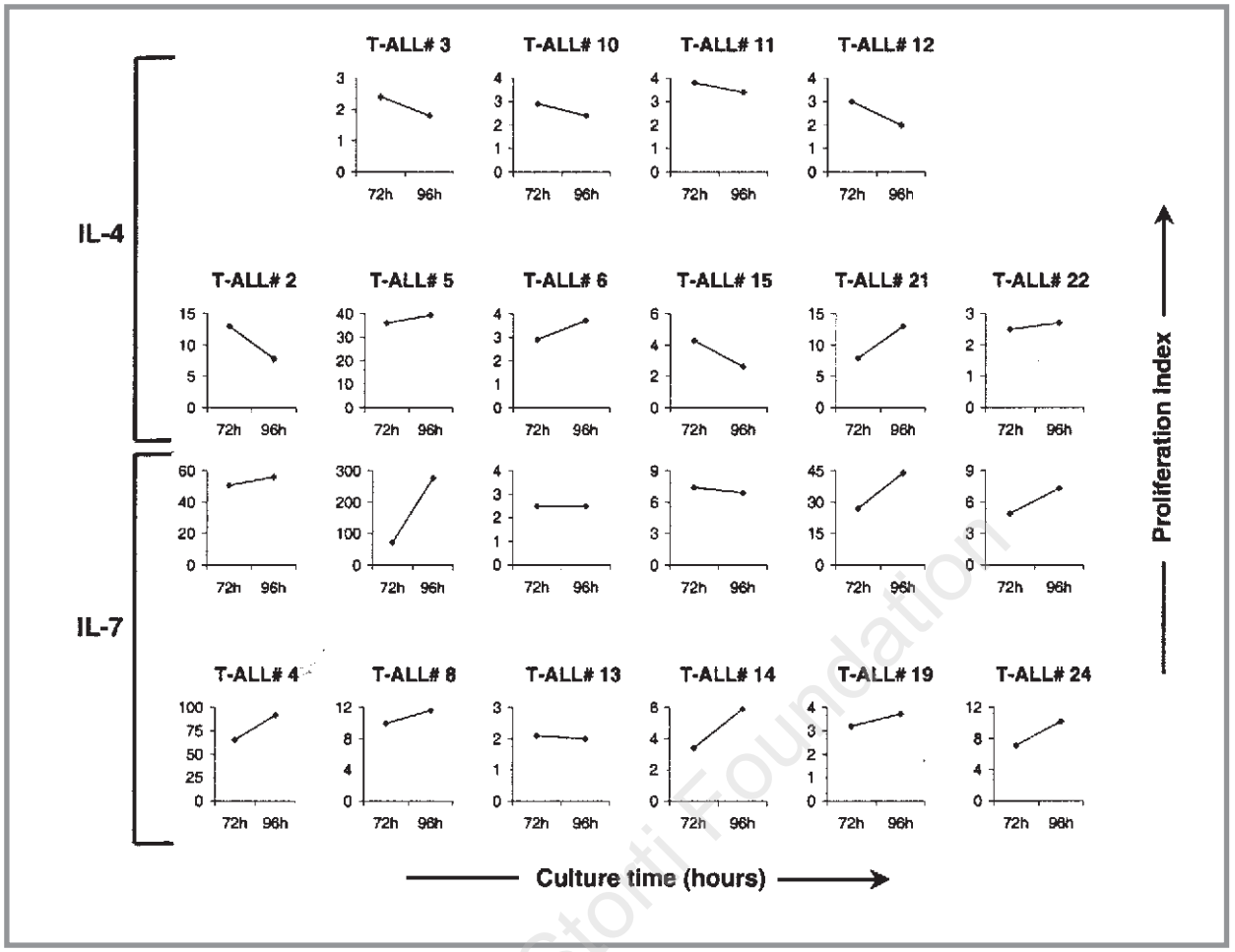

Figure 2. IL-7 and IL-4 promote different proliferation kinetics of primary T-ALL. Proliferation indices of responses to IL-4 and IL-7, at 72 and 96 hours, were calculated and are indicated for each responsive patient. Graph scales were adjusted for each individual patient to facilitate clear presentation of the data, given the high variability of responsiveness among samples. Graphics show the PI for T-ALL specimens responding uniquely to IL-4 (upper row), IL-7 (lower row) or both cytokines (middle rows, as indicated).

most patients tested, responses to IL-7 increased from 72 to 96 hours of culture (9 of 12 cases; 75%), in contrast to the effect of IL-4, which generally showed decreased proliferation at 96 hours (6 of 10 cases). Analyses at day 6 showed that IL-7 continued to induce stronger proliferative responses than did IL-4 (*data not shown*). Of note, 3 of the 4 cases (#5, 21 and 22) in which the proliferative effect of IL-4 increased at 96 hours showed a considerably higher increase with IL-7 (Figure 2, middle rows). These results indicate a differential capacity of IL-4 and IL-7 to maintain the proliferative response of primary T-ALL cells over time, and suggest that the IL-7-mediated proliferative effect is not only more robust but also more prolonged.

Stimulatory activity of IL-4 and IL-9, but not other γc-cytokines, is restricted to particular T-ALL maturation stages

Since the regulatory activities of external factors on T lymphopoiesis are generally exerted in a stage-specific manner, we investigated whether there was a correlation between the differentiation status of T-ALL cells and their responsiveness to γc-cytokines. None of the γc-cytokines showed an exclusive effect on a particular maturation stage, with all cytokines inducing proliferation of at least one sample from each T-ALL stage (Figure 1). Subsequently, we evaluated whether the intensity of the proliferative responses was also independent of the maturation stage. Responsiveness to IL-7 did not show any significant association with T-ALL maturation stages (p=0.993; Kruskal-Wallis test). Likewise, no significant associations between T-ALL stage and proliferation were observed for IL-2 and IL-15 (p=0.273 and p=0.715, respectively). Interestingly, the proliferation to IL-4 increased consistently from the most immature, pro/pre-T-ALL samples, to the most differentiated, mature T-ALL samples (p=0.035; Figure 3A). Furthermore, we observed that the proliferative responses promoted by IL-9 were significantly more robust in both pro/pre- and mature T-ALL samples than

Table 1. Synergistic and additive effects between γc signaling cytokines on proliferation of primary T-ALL cells.

T-ALL#	IL2+IL4	IL2+IL7	IL2+IL9	IL2+IL15	IL4+IL7	IL4+IL9	IL4+IL15	IL7+IL9	IL7+IL15	IL9+IL15
2	12.0 (2.8+10.3)	–	9.2 (2.5+5.6)	3.5 (2.8+2.6)	–	–	–	–	–	10.8 (7.3+2.6)
3	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–
5	ND	ND	ND	ND	–	138.9 (75.1+18.5)	88.8 (75.1+18.1)	ND	ND	ND
6	6.7 (1.0+2.6)	4.2 (1.0+1.4)	2.0 (1.0+1.4)	1.6 (1.0+1.0)	8.3 (2.6+1.4)	9.6 (2.6+1.4)	9.0 (2.6+1.0)	4.1 (1.4+1.4)	5.1 (1.4+1.0)	2.3 (1.4+1.0)
8	–	20.9 (2.8+8.7)	13.7 (2.8 + 7.2)	–	–	–	–	18.3 (8.7+7.2)	12.3 (8.7+1.6)	10.9 (7.2+1.6)
10	–	–	–	–	–	–	–	–	–	–
11	–	–	–	–	–	3.0 (1.8+1.3)	–	–	–	–
12	–	–	–	–	–	–	–	–	–	–
13	–	3.7 (1.7+2.0)	3.2 (1.7+2.2)	–	–	–	–	3.2 (2.0+2.2)	2.9 (2.0+1.2)	–
18	–	14.9 (1.5+12.8)	4.6 (1.5+2.7)	–	–	–	–	17.4 (12.8+2.7)	–	–
20	–	–	–	–	4.0 (3.0+3.5)	3.4 (3.0+1.2)	–	–	–	–
21	8.8 (7.9+1.1)	–	–	–	–	8.8 (7.9+1.2)	–	–	–	–
22	–	–	–	–	–	–	–	–	–	–
24	–	16.4 (6.5+9.5)	13.1 (6.5+5.2)	–	–	–	–	11.6 (9.5+5.2)	9.2 (7.1+2.7)	–

Values indicate proliferation index (PI) of the cytokine combination when a synergistic/additive effect was observed, as defined in the Design and Methods section. In brackets the PI of each individual cytokine. –: no synergistic or additive effect; ND: not determined.

in cortical T-ALL (p=0.026; Figure 3B). Accordingly, responsiveness to IL-9 was inversely associated with CD1a expression (p=0.007; Mann-Whitney test).

γc-signaling cytokines have additive and synergistic effects on T-ALL cell proliferation

The bone marrow and thymus contain IL-7 and IL-15 (produced by stromal cells), and likely IL-2, IL-4 and IL-9 (produced by circulating cells). Since these microenvironments in which the leukemia cells develop likely contain different γc-signaling cytokines, we analyzed the proliferative responses of primary T-ALL cells to combinations of two γc-cytokines. The scoring of synergism or of an additive effect was performed as described in Design and Methods. As shown in Table 1, all γc-cytokine combinations showed additive/synergistic effects on T-ALL proliferation. The cytokine combinations that most frequently mediated an additive or synergistic effect were IL-2 plus IL-9 (43% of the samples) and IL-2 plus IL-7 (36%). Interestingly, despite the generally high proliferation induced by IL-7, its stimulatory effects were frequently potentiated by other cytokines (plus IL-2, 36%; plus IL-9, 36%; plus IL-15, 29%). Overall, these results indicate that, when used in combination, the γc-signaling cytokines can have synergistic/additive proliferative effects on T-ALL primary cells, a property that may be relevant for the biology of T-ALL.

Discussion

The identification of molecules and signaling pathways critical for T-ALL pathophysiology should contribute to the development of novel, more effective

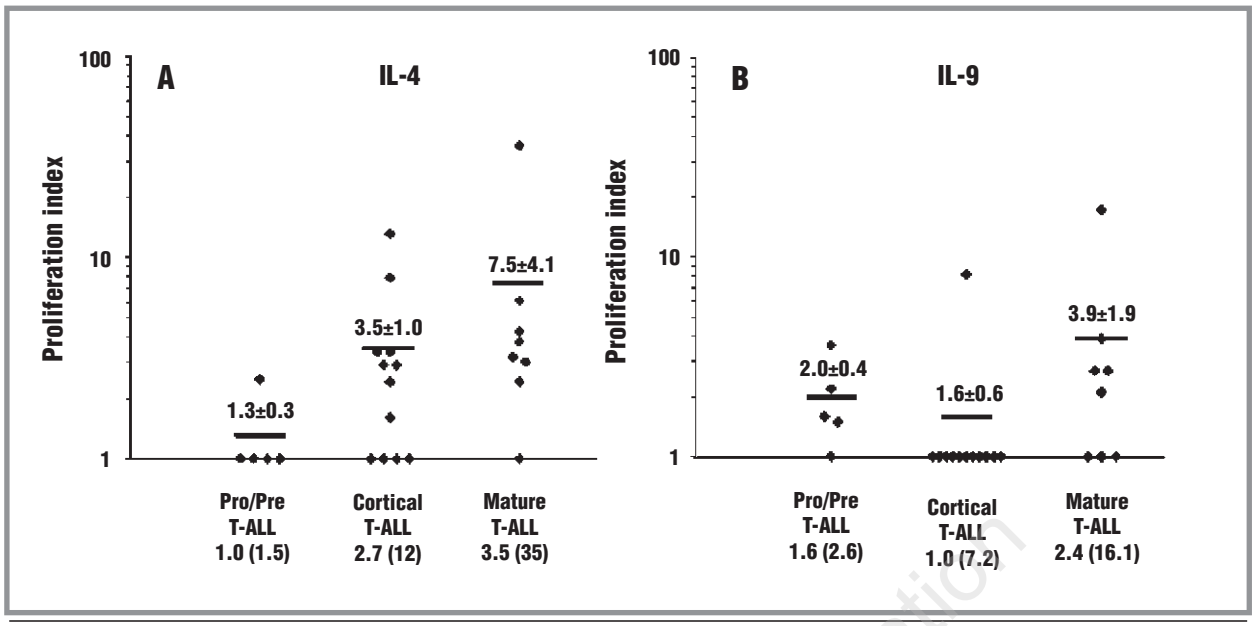

Figure 3. Proliferative responses to IL-4 and IL-9 depend on the maturation stage of the primary T-ALL cells. Each symbol indicates the PI for one T-ALL sample, at 72 hours, in response to IL-4 (A) or IL-9 (B). Data are presented on a logarithmic scale, with non-responsive cases represented as having a PI of 1. Samples were grouped according to their maturation stage, as defined by the EGIL criteria.[18] Mean PI±SEM are indicated for each maturation group. Median PI and range (in brackets) are indicated below the respective maturation group. Results are statistically significant (p=0.035 for IL-4; p=0.026 for IL-9; Kruskal-Wallis Test).

therapeutic strategies. Despite the multiple functions played by γc-signaling cytokines in normal T-cell development and biology, their role in T-ALL pathophysiology is largely unexplored. Since no systematic study addressing this question has been reported, we conducted a comprehensive analysis of the mitogenic potential of γc-cytokines on primary T-ALL cells. We observed that all γc-signaling cytokines can mediate T-ALL cell proliferation. Our data confirm previous studies showing that T-ALL blasts proliferate in response to IL-7,[1,2,13,19,22] IL-4,[1,14] and IL-2.[1,10-14] We also report, for the first time, that IL-9 and IL-15 can function as growth factors for T-ALL. The methodology employed to assess cytokine-mediated proliferation (^{3}H-thymidine incorporation) measures cell cycle entry and progression as well as putative rescue from apoptosis of cycling cells. Since primary T-ALL cells undergo spontaneous apoptosis *ex vivo*, the proliferative effects observed may be in part mediated through prevention of T-ALL cell death. However, our previous studies with IL-4[34] and IL-7[19] indicate that γc-cytokines induce cell cycle progression of primary T-ALL cells, independently of their effect on viability. Our preliminary studies with IL-2, IL-9 and IL-15 in four cases of primary T-ALL appear to confirm these observations (*data not shown*). The critical roles played by signals delivered through the γc in T-cell development[23] and their putative role in T-cell leukemogenesis,[3,17,24] suggest that targeting of γc-signaling will likely have a significant therapeutic impact. Recently, two patients with severe combined immunodeficiency who received gene therapy to correct γc deficiency developed T-cell leukemia. This may have resulted from the co-operation between constitutive expression (possibly over-expression) of the γc and the accidental, retrovirally-induced activation of a T-cell oncogene (*LMO2*).[25] These cases further underscore the possible role of γc signaling in T-ALL, and its potential as a molecular target. Some evidence suggests that during thymic development, rather than functioning as a mitogen, IL-2 participates in the elimination of autoreactive thymocytes.[5-7] However, other studies showed that human immature double negative thymocytes ($CD4^-CD8^-$) express IL-2 receptors and proliferate in response to IL-2.[26,27] In mice, IL-2 does not induce significant proliferation of immature thymocytes, despite high expression of IL-2R.[28-30] Thus, IL-2 might play a redundant role in the expansion of early thymocytes and subsequently a non-redundant function during negative selection of double positive thymocytes. However, IL-2 has also been implicated in the negative regulation of bipotential T/NK progenitors,[8] indicating that the actual role of IL-2 in thymocyte development has yet to be fully elucidated. The activity of IL-2 as a growth factor for T-ALL has been suggested by its proliferative effect *in vitro*,[1,10-14] and by the establishment of IL-2-dependent human T-ALL cell lines.[31-33] Moreover,

several studies have shown that T-ALL cells can express IL-2Rα, IL-2Rβ,[4,9-11] as well as γc.[4] The evidence that T-ALL cells secrete IL-2[12] raises the possibility that an IL-2 autocrine loop is involved in the clonal expansion of some T-ALL cases. IL-4 does not seem to play a significant role during thymocyte development, in contrast to its well-established role in skewing T-cell responses towards a Th2 phenotype. The studies regarding the effect of IL-4 on T-ALL are few and conflicting. Two early reports on small groups of patient showed that IL-4 could induce proliferation of T-ALL blasts[1,14] whereas, more recently, IL-4 was shown to prevent spontaneous apoptosis without mediating T-ALL cell proliferation.[4] Our observations support the former reports, as IL-4 induced proliferation in the majority (16 of 25) of the samples studied. Moreover, we found that IL-4 induces downregulation of p27 kip1, hyperphosphorylation of Rb, and cell cycle progression in primary T-ALL cells.[34] Several lines of evidence support the hypothesis that IL-7 plays a significant role in T-cell leukemogenesis. First, IL-7 can act as an oncogene *in vivo*, since IL-7 transgenic mice develop B and T-cell lymphomas.[3] Second, thymocytes overexpressing IL-7R have a selective advantage that contributes to proliferation and leukemogenesis.[35] Third, IL-7 is produced by bone marrow and thymic stroma and therefore it is present in the microenvironments where the leukemic cells develop. Hence, IL-7 has the potential to directly modulate T-ALL growth. In fact, thymic epithelial cells have been shown to produce IL-7, thereby promoting the survival of T-ALL cells.[36] Fourth, T-ALL cells express the receptor for IL-7[1,2,20,37,38] and the anti-apoptotic effect of IL-7 on T-ALL[4,22] seems to correlate with IL-7Rα surface expression.[4] Finally, various groups have demonstrated that IL-7 stimulates T-ALL proliferation *in vitro*.[1,2,13,20-22] However, these studies were based on small numbers of samples. Here, we confirmed these results using a larger number of T-ALL cases. Our demonstration that IL-7 is the γc-signaling cytokine that induces more robust proliferative responses and in the largest number of patients (72% of the samples) suggests that it may play a role in supporting T-ALL clonal expansion. Accordingly, we were able to establish an IL-7-dependent cell line from a pediatric patient with T-ALL; this cell line shares the phenotypic, functional and signaling properties of primary leukemic cells.[39]

We show, for the first time, that IL-9 and IL-15 can promote the proliferation of T-ALL cells. The intensity of T-ALL proliferation in response to these cytokines was equivalent to that induced by IL-2. IL-9 was suggested to be critical for the early stages of human T-cell development, and both IL-9 and its receptor are present in human thymocytes.[40] Moreover, IL-9 prevents dexamethasone-induced T-cell apoptosis.[41] A link between IL-9 and T-cell malignancies has also been suggested. IL-9 has clear *in vitro* anti-apoptotic[15] and proliferative[16] effects in thymic T-cell lymphomas. Additionally, IL-9 transgenic mice have increased incidence of thymic T-cell lymphomas, suggesting that deregulated IL-9 expression could be involved in the development of some T-cell malignancies.[24] Normal thymocytes do not seem to require IL-15 for their development, although thymic cellularity is somewhat reduced in IL-15R-/- mice.[42] Interestingly, studies with double knockout mice have demonstrated that IL-15 plays a redundant role with IL-7 in early thymic T-cell development,[43] and IL-15 mRNA is expressed in the thymic epithelial[8] and bone marrow stromal cells.[44] Moreover, long-term bone marrow stromal cell cultures secrete IL-15.[44] Since IL-15 transgenic mice develop leukemia,[17] and IL-15 is likely produced in the microenvironments in which the leukemic cells arise, it is conceivable that deregulated expression of IL-15 might also influence some T-ALL cases.

A possible association between γc-signaling cytokine-induced proliferation and T-ALL maturation stage has not been previously addressed. However, Karawajew *et al.* demonstrated that IL-7 prevented spontaneous apoptosis preferentially in T-ALL cells of cortical and mature phenotypes.[4] In our patient population, we did not observe a significant association between responsiveness to IL-7 and T-ALL maturation stage. Likewise, IL-2 and IL-15 do not seem to stimulate preferentially T-ALL cells of any particular developmental stage. We cannot exclude the possibility that the observed lack of statistically significant associations may be due to limited number of cases available for this study. In contrast, responsiveness to IL-4 progressively increased with the acquisition of maturation markers. Interestingly, cortical T-ALL cells, characterized by the expression of CD1a, were mostly unresponsive to IL-9. Some studies have shown that CD1a-positive patients have significantly better responses to prednisone and a more favorable outcome than do patients with other immunophenotypic T-ALL subsets.[45,46] Based on our studies, it will be interesting to evaluate whether responsiveness to IL-9 may serve as an indicator of poor prognosis for T-ALL patients treated with conventional therapies. Importantly, expression of the oncogenic transcription factor LYL1 in T-ALL is associated with increased expression of IL-9R and an immature CD1-negative phenotype.[47] A considerable portion of the IL-9-responsive T-ALL samples in our study shared the same phenotype, raising the interesting question of whether IL-9 can stimulate the expansion of $LYL1^+$ T-ALL cells *in vivo*. Previous studies support a role for cytokine synergisms in normal T-cell development and in malignant cells. IL-7 and IL-2 were shown to exert a synergistic effect on the proliferation of immature $CD4^-CD8^-$ double

negative thymocytes.[48,49] Also, IL-2 has been shown to synergize with Steel factor in the T-ALL cell line PER-4,[31] suggesting that IL-2 may act in concert with other cytokines to regulate T-ALL cell function. In human early T-cell development, IL-9 has been proposed to complement the effect of IL-7 in overcoming a minimum threshold of activation essential for optimal T-cell production.[40] Interestingly, a recent study has demonstrated that IL-9 acts synergistically with IL-7 to promote cell proliferation of pre-malignant thymocytes during the process of lymphomagenesis in irradiated mice.[50] Our data on human malignant T-cells confirms the ability of IL-9 to act synergistically with IL-7. The demonstration that γc-signaling cytokines can mediate additive and synergistic effects on T-ALL is likely relevant for the biology of this cancer. This is particularly significant for IL-7, whose generally robust proliferative effects can be potentiated by IL-2, IL-4, IL-9 or IL-15. Since IL-7 seems to play a significant role in leukemogenesis,[3] and triggers multiple signaling pathways regulating cell survival, proliferation and growth in T-ALL (PI3K/AKT, JAK/STAT, MEK/Erk, mTOR),[2,19,39] it will be important to dissect the mechanisms by which this cytokine cooperation is exerted, and its biological relevance. In conclusion, we show that the γc-cytokines IL-2, IL-4, IL-7, IL-9 and IL-15 can promote proliferation of primary T-ALL cells, and that their individual activities can be potentiated by additional γc-triggered signals. In the light of these observations, and the putative role of IL-7 in leukemogenesis, this study suggests that γc signaling may have a significant function in T-ALL, and may constitute a valid target for therapeutic intervention.

JTB, VAB and AAC designed the study and performed the analysis and interpretation of the results; JTB, TDK and AS performed the experiments; JTB and AAC drafted the manuscript; all authors critically revised the manuscript and gave their final approval of the version to be submitted. The authors reported no potential conflicts of interest.

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