# Thymic epithelial cells promote survival of human T-cell acute lymphoblastic leukemia blasts: the role of interleukin-7

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*Background and Objectives*. T-cell lymphoblastic leukemia (T-ALL) cells originate within the thymus from the clonal expansion of T cell precursors. Among thymic stromal elements, epithelial cells (TEC) are known to exert a dominant inductive role in survival and maturation of normal, immature T-cells. In this study we explored the possible effect of TEC on T-ALL cell survival and analyzed the role of interleukin-7 (IL-7) within the microenvironment generated by T-ALL-TEC interactions.

*Design and Methods*. T-ALL blasts derived from 10 adult patients were cultured with TEC obtained from human normal thymuses. The level of blast apoptosis was measured by annexin V-propidium iodide co-staining and flow cytometry. The proliferative response of leukemic cells to interaction with TEC was evaluated by thymidine incorporation at various time intervals of culture. To assess the role of IL-7, lympho-epithelial co-cultures were carried out in the presence of anti-IL-7 or anti IL-7R blocking antibodies and the level of apoptosis of T-ALL blasts was analyzed.

*Results*. When T-ALL cells were cultured in the presence of TEC monolayers, the percentage of viable cells increased significantly and this survival was sustained with time in culture. In addition, the interaction with TEC induced a considerable proliferative response in T-ALL cells (15-fold greater than that of the control cells after 7 days of culture). The presence of IL-7 or IL-7R blocking antibodies in lympho-epithelial co-cultures consistently reduced the TEC-mediated apoptosis inhibition in T-ALL blasts (70% decrease).

*Interpretation and Conclusions*. These results point to the role of thymic epithelium in the regulation of T blast survival. In addition, they show that interaction between IL-7 and its receptor has the major role in modulating T-ALL survival within the microenvironment generated by the T-ALL/TEC interaction.

Key words: human, thymus, T-ALL, TEC, interleukin-7.

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T-cell acute lymphoblastic leukemia (T-ALL) is a malignant disease resulting from the clonal proliferation of T lymphoid precursors. It accounts for about 15% of all ALL cases in children and 20-25% in adults.[1,2] T-ALL is thought to originate inside the thymus and leukemic cells express phenotypic features corresponding to distinct maturational stages of thymocyte development: early (stage I), intermediate (stage II), or late (stage III).[2,3]

The thymus is the main site where bone marrow (BM)-derived stem cells differentiate into mature, immunocompetent T lymphocytes.[4,5] The internal stromal framework of the thymus is composed of epithelial cells, interspersed with BM-derived macrophages and dendritic cells, surrounded by extracellular matrix to form an intralobular meshwork filled with developing T lymphocytes.[6-8] The thymic epithelium is composed of multiple, distinct cellular subsets, characterized by tonofilaments and interconnected by desmosomes. Three broad classes of epithelium have been identified by using monoclonal antibodies to antigen determinants: subcapsulae/subtrabeculae/perivascular, cortex, and medulla.[6] Although distinct functions of the different subsets have not been fully elucidated, it is known that thymic epithelial cells (TEC) exert a pivotal role in the homing, intrathymic migration, and differentiation of thymocytes through the release of cytokines, the secretion of extracellular matrix components, and the establishment of adhesive interactions.[9-13] Furthermore, *in vitro* experiments using co-cultures between thymocytes and TEC or mouse fetal thymic organ culture (FTOC), indicate that adhesive interactions with TEC or with factors secreted by TEC can protect thymocytes from apoptosis.[14,15] A recent report indicates that mouse FTOC can induce the proliferation of blasts from T-ALL patients, thus suggesting that thymic stromal elements may also have a role in regulating the growth of malignant thymocytes.[16]

Interleukin-7 (IL-7) has a critical, non-redundant role in normal T-cell development. IL-7 is secreted by thymus and BM stromal cells and exerts its activity by signaling through a receptor complex consisting of the IL-7Rα chain and γc. Mice deficient in IL-7 have a drastically reduced number of thymocytes and a defect in the developmental transition from immature to T-cell committed thymocytes.[17] Similarly, mice deficient in IL-7R show an early defect in lymphopoiesis, and the few mature T cells which do develop are functionally impaired.[18] The critical role of IL-7 for early T-cell devel-

opment was also demonstrated in chimeric human-mouse thymus organ culture models in which antibodies blocking IL-7 and IL-7R were used.[19] Furthermore, IL-7 is involved in leukemogenesis as IL-7 transgenic mice develop lymphoid tumors.[20] Different studies have indicated a role for IL-7 in regulating survival and cell cycling of blasts in T-ALL patients, thus suggesting that IL-7 may regulate the expansion of malignant cells.[21-27] However, in FTOC systems the growth of T-ALL cells appears to be independent from IL-7.[16] Thus, the putative role of IL-7 as a survival factor for T-ALL blasts in the thymus has not yet been fully elucidated.

In this report, we show that TEC reduce spontaneous apoptosis and induce proliferation in cells from T-ALL patients. Furthermore, the functional blockage of IL-7 or IL-7R reduces TEC-mediated apoptosis inhibition in T-ALL. We propose that TEC have a functional role in modulating the survival of T-ALL blasts by a mechanism mainly dependent on the interaction between IL-7 and its receptor.

## Design and Methods

### *Cells*

Peripheral blood samples were collected, after informed consent, from 10 adult patients with newly diagnosed T-ALL. Mononuclear cells were isolated by Lymphoprep density gradient centrifugation (Nicomed, Oslo, Norway). In two co-culture experiments (cases 1 and 3), cells were used immediately after preparation whereas cryopreserved samples were employed, immediately after thawing, in the other cultures. Comparative experiments showed no significant differences between results obtained from fresh or thawed samples (*data not shown*). Before use, the cells' viability consistently exceeded 90% in each sample, as assessed by propidium-iodide (PI) dye exclusion.

Thymic epithelial cell cultures were derived, after informed consent, from normal thymuses of children (< 5 years of age) undergoing cardiac surgery, as previously described.[28,29] Briefly, thymus specimens were minced and treated with a 0.05% trypsin-0.01% EDTA solution at 37°C for 3 hours. Cells were collected every 30 minutes, pooled, plated onto lethally irradiated 3T3-J2 cells[30] (kindly provided by Dr H Green, Harvard Medical School, Boston, MA, USA) at $2.5\times10^4/cm^2$, and cultured in a humidified atmosphere of 5% $CO_2$, in growth medium composed of the following: DMEM (Dulbecco's modified Eagle's medium) and Ham's F12 medium (3:1 mixture), 10% FCS (fetal calf serum), 5 μg/mL insulin, 5 μg/mL transferrin, 0.18 μM adenine, 0.4 μg/mL hydrocortisone, 0.1 nM cholera toxin, 2 nM triiodothyronine, 10 ng/mL epithelial growth factor (EGF), 4 mM glutamine, and 50 IU/mL penicillin-streptomycin. Confluent TEC primary cultures were detached by trypsin-EDTA treatment, plated on a 3T3-J2 feeder layer and expanded to confluent secondary cultures in growth medium. TEC destined to co-culture experiments were derived from secondary cultures, already devoid of 3T3-J2 cells, and grown to confluence. Media were purchased from Seromed (Berlin, Germany), EGF from Austral Biological (San Ramon, CA, USA), and supplements from Sigma-Aldrich (Milan, Italy).

The HeLa human epithelial-like cell line was cultured in DMEM (GIBCO), 10% FCS, L-glutamine, and antibiotics.

### *Co-cultures and antibody treatment*

TEC or HeLa cells destined to co-cultures were grown to a confluent state. For proliferation assays, epithelial monolayers were sublethally irradiated 24 hours before use whereas non-irradiated TEC were used for apoptosis analysis of blasts. Immediately before experiments, the medium from epithelial monolayers was removed and the cells washed extensively. T-ALL blasts were added to epithelial monolayers at a 10:1 blast:epithelial-cell ratio and cultured in a humidified atmosphere of 5% $CO_2$, in medium composed of DMEM and Ham's F12 medium (3:1 mixture), 10% FCS, 5 μg/mL insulin, 5 μg/mL transferrin, 0.18 μM adenine, 4 mM L- glutamine, and 50 IU/mL penicillin-streptomycin. Control T-ALL blasts were cultured in the same conditions but in the absence of epithelial monolayers. At the end of co-culture, cells were harvested by vigorous pipetting. The lack of residual cells in the wells was assessed by phase contrast microscopy. The T-cell nature of the cells harvested from co-cultures was assessed by immunofluorescence with anti-CD5 monoclonal antibody and flow cytometry (*data not shown*).

For blocking experiments, neutralizing concentrations (10 μg/mL) of antibodies recognizing functional epitopes of IL-7 (rabbit polyclonal, Biosource International, Camarillo, CA, USA) or IL-7R (clone R34.34, Instrumentation Laboratory, Milan, Italy) were added to the T-ALL/TEC co-cultures at the start of the assay.

### *Immunophenotype analysis*

Immunophenotype analysis of T-ALL blasts and TEC was performed by direct immunofluorescence and flow cytometry with a FACScalibur instrument (Becton-Dickinson, San José, CA, USA). For intracellular CD3 and terminal deoxynucleotidyl transferase (TdT) analysis, cells were permeabilized with Fix&Perm reagents (Caltag Laboratories, Burlingame, CA, USA) and treated according to the manufacturer's instructions. The following monoclonal antibodies were used for surface or intracellular analysis: FITC-conjugated anti-CD2 (clone S5.2), anti-CD5 (clone L17F12), anti-CD7 (clone 4H9),

anti-CD8 (clone SK1); PE-conjugated anti-CD1a (clone SK9), anti-CD3 (clone SK7), anti-CD4 (clone SK3), all from BD Pharmingen (San Diego, CA, USA); FITC-conjugated anti-TdT (clone HT-6) and cy5-conjugated anti-CD3 (clone UCHT-1) from DAKO (Glostrup, Denmark); and PE-conjugated anti-CDw127 (IL-7Rα, clone R34.34) from Instrumentation Laboratory. Conjugated isotype-matched, non-reactive monoclonal antibodies were used as controls.

### *Annexin staining*

The exposure of phospholipid phosphatidylserine on the plasma membrane of apoptotic cells was assessed by the binding of FITC-conjugated annexin V on the membrane surface. Cells were collected at the indicated time of culture and stained with annexin-FITC in conjunction with propidium iodide, according to the recommended protocol of the manufacturer (Bender Med System, Vienna, Austria). Stained cells were analyzed with a FACScalibur flow cytometer (Becton Dickinson).

### *Proliferation assay*

Cell proliferation assays were performed in triplicate in 96-well culture plates (Costar, Cambridge, MA, USA). T-ALL blasts were co-cultured with irradiated epithelial layers for 7 days. Cells were pulsed with 1 μCi/well of (methyl-$^3$H) thymidine for 18 hours at the indicated time points of culture, and harvested onto a paper filter. Radioactivity was measured by a liquid scintillation counter (Beckman Instruments Inc). Results were expressed either as absolute cpm or proliferation index. This latter index is calculated as cpm of stimulated cells/cpm of untreated cells.

### *IL-7 production*

Cell supernatants were assayed for IL-7 production using a high sensitivity ELISA kit, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Optical density values were plotted on the standard curve and expressed as picograms/mL per $10^6$ cells.

### *Statistical analysis*

Values are expressed as mean ± SD. Differences (*p* values) were evaluated using the 2-tailed Student's t-test. Differences were considered statistically significant for *p* values <0.05.

## Results

### *Thymic epithelial cells protect T-ALL cells from spontaneous apoptosis*

The ability of TEC to regulate the spontaneous apoptosis of leukemic cells was determined by co-culturing T-ALL blasts *in vitro* with thymic epithelial monolayers.

**Table 1. Characteristics of the cases of T-lineage ALL.**

| *No.* | *Age (yr)* | *Immunophenotype* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *CD1a* | *CD2* | *SCD3** | *CyCD3^CD4/CD8§* | *Stage* | |
| *1* | 32 | 15% | 96% | Neg | 95% | 96% | II |
| 2 | 19 | 87% | 96% | 85% | Neg | 73% | II |
| 3 | 32 | 25% | 96% | 50% | 34% | 87% | II |
| 4 | 41 | Neg | 60% | Neg | 89% | Neg | I |
| 5 | 33 | 14% | 97% | Neg | 89% | 95% | II |
| 6 | 17 | 54% | 100% | 97% | Neg | Neg | II |
| 7 | 25 | Neg | Neg | Neg | 93% | Neg° | I |
| 8 | 33 | 10% | 88% | Neg | 96% | Neg | II |
| 9 | 38 | 83% | 27% | 88% | 10% | Neg | II |
| 10 | 24 | Neg | Neg | Neg | 99% | Neg | I |

*In all cases, more than 90% of blasts were positive for CD5, CD7 and TdT. *Surface CD3; ^Cytoplasmic CD3; §Co-expression of CD4 and CD8; °thirty percent of blasts were single positive for CD8.*

The blasts used in this study were derived from enriched leukemic cells isolated from adult patients with T-ALL. In each of the 10 cases analyzed, more than 90% of blasts were positive for the pan-T-cell markers CD7 and CD5 as well as the DNA polymerase TdT. Six patients were CD3-negative at the surface level and expressed cytoplasmic CD3 in the majority of blasts. One patient partially expressed both surface and cytoplasmic CD3 and the other three showed a more mature phenotype (surface CD3$^+$). On the basis of these features, patients were classified according to maturational stages of T-ALL,[31] overall corresponding to a cortical thymocyte-like phenotype for all cases except for n. 4, 7, and 10, which corresponded to an early *double negative* or *immature single positive* stage (Table 1).

The TEC used in co-culture experiments were derived from 5 independent donors. All cultures were performed with morphologically identical and confluent epithelial cell layers. The epithelial origin of thymic stromal cells obtained with our procedure has been established by the presence of tight junctions[29] and positive staining with anti-cytokeratin 19 (*data not shown*). Furthermore, the expression of an antigen determinant which characterizes the medullary epithelium (namely MR6, *see ref. 6*) and the contemporary absence of a determinant expressed by cortical epithelium (namely MR19, *see ref. 6*) suggest that the TEC used

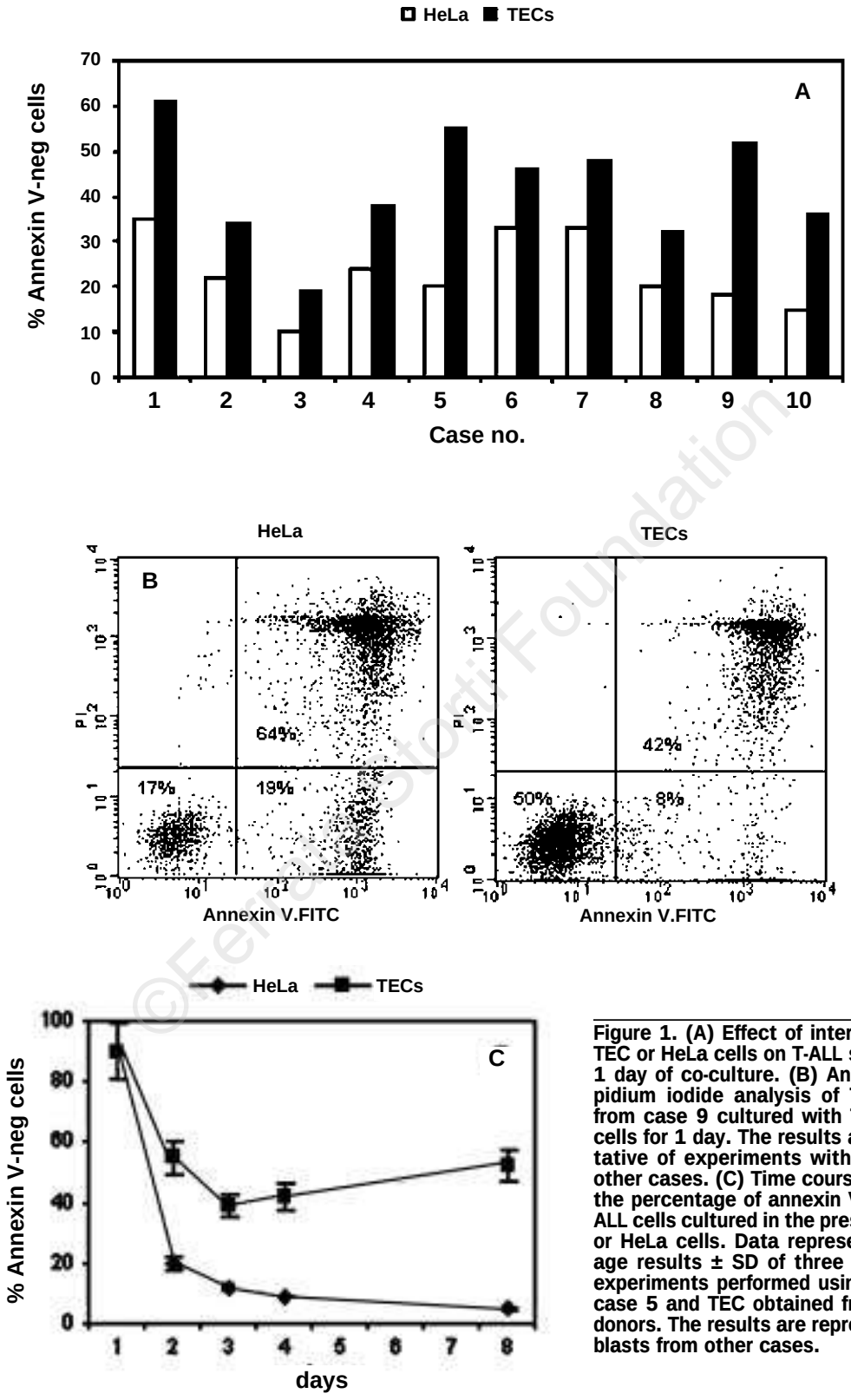


**Figure 1. (A) Effect of interactions with TEC or HeLa cells on T-ALL survival after 1 day of co-culture. (B) Annexin V/propidium iodide analysis of T-ALL blasts from case 9 cultured with TEC or HeLa cells for 1 day. The results are representative of experiments with blasts from other cases. (C) Time course analysis of the percentage of annexin V-negative T-ALL cells cultured in the presence of TEC or HeLa cells. Data represent the average results ± SD of three independent experiments performed using cells from case 5 and TEC obtained from different donors. The results are representative of blasts from other cases.**

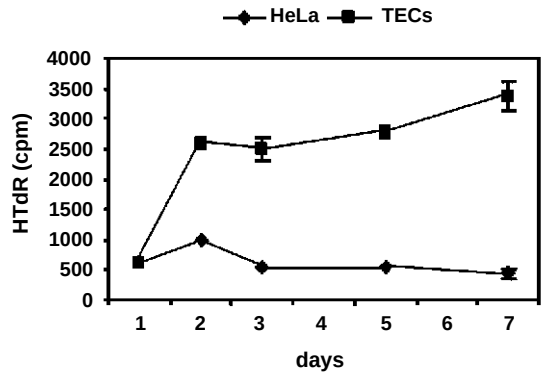


**Figure 2. Time course analysis of HTdR-incorporation microassay in T-ALL cultured in the presence of TEC or HeLa monolayers. Data are expressed as mean values ± SD of 6 independent experiments (cases #1, 2, 3, 4, 5, and 6).**

in this study derived from the cortical area of the thymic lobule (*data not shown*).

The survival of leukemic cells from all patients cultured with TEC was significantly greater than that of leukemic cells cultured in medium alone or in the presence of HeLa epithelial cells (Figure 1A). The mean ± SD of increased survival in TEC culture with respect to HeLa cells was 1.99-fold±0.62 (range 1.39-2.94-fold, n=10, $p<0.001$) after 24 hours of co-culture. Comparable levels of cell viability were attained when cells from the same patient were cultured with TEC from different donors (*data not shown*). Figure 1B shows the flow cytometric analysis of cells from case #9. As shown in Figure 1C for cells from case #5, the level of increased cell viability was maintained with time in culture.

### *TEC induce proliferation in T-ALL blasts*

We next evaluated the proliferative response of leukemic cells to stimuli deriving from TEC. To this aim blasts derived from T-ALL patients (cases #1, 2, 3, 4, 5 and 6) were co-cultured with sublethally irradiated TEC. DNA synthesis was then evaluated by thymidine incorporation at various time intervals of culture. As shown in Figure 2, in all analyzed leukemic blasts the interaction with TEC resulted in a clear-cut increased DNA synthesis compared when the blasts were cultured with HeLa cells. The mean values ±SD of the proliferation index after 7 days of culture on TEC or HeLa cells were 15.2±3.42 and 2.65±0.735, respectively (n=6). In contrast to the response of primary T-ALL cells to TEC, no proliferation was observed in thymocytes or purified T lymphocytes derived from normal donors (*data not shown*). After 7 days of co-culture with TEC, more than 90% of cells from all cases expressed CD5 molecule, as determined by immunostaining and flow cytometry (*data not shown*), thus confirming the blast nature of the proliferating cells.

### *The IL-7/IL-7Rα system is required for the TEC- mediated inhibition of apoptosis in T-ALL*

We investigated the possible role of IL-7 in the TEC-mediated enhancement of T-ALL survival. When T-ALL blasts were cultured in the presence of IL-7, significantly increased cell viability was detected by annexin V/propidium iodide staining and flow cytometry analysis, a finding consistent with previous results[26,27] (*data not shown*).

The ability of TEC to produce soluble IL-7 was evaluated by analyzing supernatants collected from 3 independent cultures at days 1, 3, and 7. TEC monolayers secreted IL-7 into the medium and the production increased with time in culture (0.92±0.014 pg/mL at day 1, 1.50±0.007 pg/mL at day 3, 6.58±0.021 pg/mL at day 7, for a representative TEC culture). Comparative experiments showed no significant differences between the amounts of IL-7 produced by irradiated or non-irradiated TEC (*data not shown*). In contrast, T-ALL cells did not secrete detectable amounts of IL-7 (*data not shown*). All analyzed blasts (cases n. 3, 6, 7, 8, 9, and 10) expressed high and variable levels of IL7-Rα (CDw127) (*data not shown*).

The functional role of IL-7/IL-7R-mediated signals in the apoptosis inhibition induced by TEC was then evaluated by co-culturing T-ALL and TEC in the presence of either IL-7 or IL-7Rα blocking antibodies. In all analyzed cases (case n. 3, 5, 6, 7, and 10) blockage of the IL-7/IL-7R interaction consistently reduced the extent of apoptosis inhibition observed in T-ALL blasts cultured with TEC, as determined by annexin V/propidium iodide analysis. The mean percent reduction of apoptosis inhibition was 68%±4 (range 63-74%, n=5) in the presence of IL-7 blocking antibody and 74%±9 (range 61-87%, n=5) when IL-7R was functionally impaired. In contrast, the level of apoptosis inhibition was unmodified when the T-ALL blasts were co-cultured in the presence of either an irrelevant rabbit polyclonal antibody or mouse monoclonal IgG used as controls of anti-IL-7 or anti-IL-7R antibodies, respectively (Figure 3).

## Discussion

In this report, we show that epithelial cells derived from human normal thymus are able to increase survival and induce proliferation of blasts from T-ALL patients. TEC obtained with our experimental procedure form organized monolayers that display a homogeneous morphology, retain their

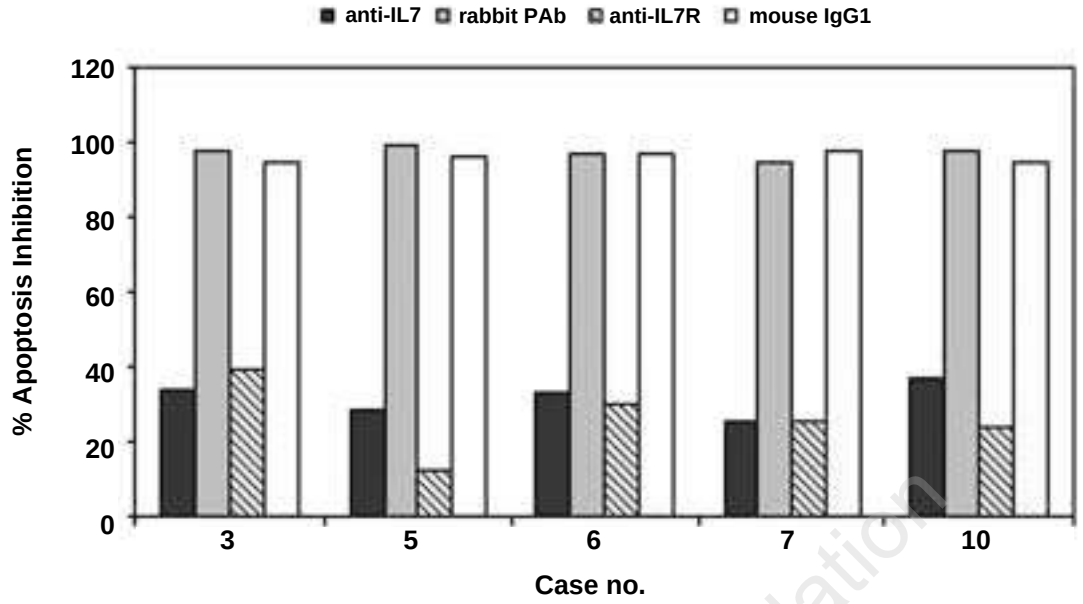


**Figure 3. Effect of IL-7 or IL-7Rα blocking antibodies on the TEC-mediated increased survival of T-ALL. T-ALL cells were cultured on TEC monolayers in the presence or absence of anti-IL-7, IL-7Rα or control antibodies for 24 hours. The level of viable cells was detected by annexin V / propidium iodide staining and flow cytometric analysis. Data are expressed as percent reduction of the apoptosis inhibition achieved when T-ALL cells are cultured on TEC, in the absence of blocking antibodies.**

surface phenotype throughout the various culture passages and produce their own matrix. We have previously shown that signals delivered by integrin recruitment after adhesion with thymocytes up-regulate TEC interleukin-6 production and promote cell survival.[29,32] Thus, the TEC used in this study preserve functional thymic epithelial properties and the ability to modulate them upon interaction with normal thymocytes. We show here that cultured TEC are able to produce IL-7 constitutively. This cytokine is known to be secreted by stromal components of the thymus and bone marrow.[33,34] These data extend our previous results and show that TEC cultures can reproduce some of the inductive elements which are active *in vivo* inside the microenvironment in which malignant T-cells develop.

The ability of TEC monolayers to regulate T-blast survival requires the IL-7/IL-7R interaction, as the application of IL-7 or IL-7Rα blocking antibodies during *in vitro* lympho-epithelial cultivation reduces the inhibition of apoptosis in T-ALL blasts. This finding is in agreement with recent reports indicating a role for IL-7 in inducing both survival and proliferation of T-ALL.[26,27] In addition, our data highlight a role for IL-7 in the regulation of T-ALL survival within a lympho-epithelial co-culture system aimed at partially reproducing the cell interactions occurring *in vivo* inside the thymus. However, the block of apoptosis inhibition observed in the presence of IL-7/IL-7R functional impairment is incomplete. This suggests the existence of other mechanisms potentially involved in the modulation of T-ALL survival acting in cooperation with IL-7 in the thymus. In addition, a recent report shows that the growth of human T-ALL cells in mouse FTOC is independent of IL-7.[16] Furthermore, there are recent descriptions of a considerable proportion of samples unresponsive to IL-7.[26] Taken together, these findings suggest that alternative signals could be active in different cell culture systems and/or in IL-7 unresponsive T-blasts. Various soluble and adhesive elements acting as deliverers of survival signals in the thymus could be involved in modulating T-ALL survival.[8,9,13,15] Among them, adhesion molecules are of particular interest both for their signaling properties and their ability to co-operate with growth factors in the regulation of many cellular processes.[35,36] In this context, the involvement of LFA-1 and E-selectin adhesion molecules, recently described for the T-ALL survival sustained by BM stroma, is noteworthy.[37] Based on these findings, it could be argued that T-ALL blast survival within the thymus might be regulated by complex signaling pathways.

In contrast to T-ALL blasts, normal thymocytes used as controls failed to proliferate when co-cultured with TEC. As the bulk thymocytes used in this study are at the double positive stage, this finding

is consistent with previous reports indicating that thymocyte proliferative potential is high in immature double negative cells and that it decreases as thymocytes move towards the double positive stage, when it ceases.[38,39] In agreement with this, TEC-mediated proliferation has been described overall for early thymocytes.[13,15] Furthermore, this finding is consistent with the role of IL-7 in the microenvironment generated by TEC cultures. Indeed, various *in vivo* studies demonstrate that the IL-7/IL-7R interaction has a critical role in the development and expansion of early CD4-CD8- thymocyte precursors as well as in the intrathymic expansion of positively selected thymocytes.[19,34,40,41] Consistently, *in vitro*, thymocytes at the early stage of thymic development are responsive to IL-7; they lose this ability to respond to IL-7 after entering the subsequent cortical, double-positive ($CD4^+/CD8^+$) stage then regain IL-7-responsiveness during the post-selection phase.[9,34] T-ALL blasts used in this study showed phenotypic characteristics corresponding to cortical ($CD4^+/CD8^+$ and/or $CD1a^+$) or early ($CD7^+$, cytoplasmic $CD3^+$, and $TdT^+$, in the absence of other T-lineage markers) immature T cells. This observation is suggestive of an IL-7 responsiveness profile in T-ALL blasts which evades the responsiveness pattern of normal counterpart cells and resembles that of early thymocytes.

To exclude the possibility that T-ALL-proliferation observed upon interaction with TEC could be ascribed to contamining T-cells in the peripheral blood T-ALL samples, we evaluated the proliferative response of T-cells upon culturing with TEC. When cultured in the presence of TEC monolayers, peripheral T cells from normal donors failed to proliferate. A great deal of interest has recently arisen about the effects of IL-7 on homeostatic proliferation of peripheral T-cells. IL-7 can enhance the homeostatic proliferation of both naive and memory $CD8^+$ T-cells[42] and synergizes with IL-15 in driving the proliferation of memory, but not naive, $CD4^+$ T cells.[43] However, consistently with our findings, *in vitro* IL-7 is poorly mitogenic on its own, in the absence of factors that co-operate in driving cell-cycle progression.[43]

T-ALL cells from different patients show variable values of responsiveness to TEC. As TEC derived from different donors induced comparable effects on blasts from the same patient, we can reasonably exclude that this variability is due to functional heterogeneity among thymic epithelial cultures. A similarly variable sensitivity has been described for IL-7, in a recent study including a large number of pediatric T-ALL samples.[26] This study, by Karawajew *et al*., showed that apoptosis inhibition mediated by IL-7 correlated with the expression values of the IL-7Rα chain. In our study, we observed a tendency to correlation between the responsiveness levels to TEC and the values of IL-7R expression (*data not shown*). A higher number of cases will be required in order to establish a statistically significant association. On this basis, we can only suggest that the variable responsiveness of T-ALL blasts to TEC could be, at least partially, attributed to different levels of IL-7Rα detected in blasts.

The molecular pathways of IL-7 signaling and its influence on cell survival are poorly elucidated. However, the early molecular events triggered by IL-7R have been shown to be directly linked to a functional tyrosine-specific protein kinase pathway and involve the activation of phosphatidylinositol 3-kinase (PI-3K) and inositol phospholipid turnover pathways in human fetal thymocytes and T-ALL blasts.[21,44] Various transcription factors are subsequently activated.[45,46] Recent research indicates that the cyclin-dependent kinase (cdk) inhibitor $p27^{kip1}$ plays a key role in regulating both blast survival and proliferation mediated by IL-7R. The IL-7 signaling pathway directly downregulates the activity of $p27^{kip1}$, thereby inducing cell cycle progression and upregulation of bcl-2 in T-ALL blasts.[27] Further studies will be required to define whether molecular pathways triggered by IL-7/IL-7R could be involved in the signaling leading to enhanced survival and proliferation of T-ALL upon interaction with TEC.

In conclusion, we show that the microenvironment generated *in vitro* by the interaction between TEC and T-ALL blasts promotes the survival and proliferation of T-ALL blasts by a mechanism mainly dependent on the IL-7/IL-7R interaction. These findings suggest a functional role for the thymic microenvironment in the acquisition of selective growth advantage of leukemic cells.

## References


1. Rivera GK, Crist WM. Acute lymphoblastic leukemia. In: Handin RI, Stossel TP, Lux SE, editors. Blood: Principles and Practice of Hematology. Philadelphia: JB Lippincott Company. 1995. p. 743-59.
2. Uckun FM, Sensel MG, Sun L, Steinherz PG, Trigg ME, Heerema NA, et al. Biology and treatment of childhood T-lineage acute lymphoblastic leukemia. Blood 1998;91:735-46.
3. Foa R, Migone N, Gavosto F. Immunological and molecular classification of acute lymphoblastic leukemia. Haematologica 1986;71:277-85.
4. Sprent J, Lo D, Gao EK, Ron Y. T cell selection in the thymus. Immunol Rev 1988;101:173-90.
5. van Ewijk W. T-cell differentiation is influenced by thymic microenvironments. Annu Rev Immunol 1991;9:591-615.
6. Boyd RL, Tucek CL, Godfrey DI, Izon DJ, Wilson TJ, Davidson NJ, et al. The thymic microenvironment. Immunol Today 1993;14:445-9.
7. Screpanti I, Modesti A, Gulino A. Heterogeneity of thymic stromal cells and thymocyte differentiation: a cell culture approach. J Cell Sci 1993;105:601-6.
8. Anderson G, Moore NC, Owen JJ, Jenkinson EJ. Cellular interactions in thymocyte development. Annu Rev Immunol 1996;14:73-99.
9. Zlotnik A, Moore TA. Cytokine production and requirements during T-cell development. Curr Opin Immunol 1995;7:206-13.
10. Utsumi K, Sawada M, Narumiya S, Nagamine J, Sakata T,

Iwagami S, et al. Adhesion of immature thymocytes to thymic stromal cells through fibronectin molecules and its significance for the induction of thymocytes differentiation. Proc Natl Acad Sci USA 1991 88:5685-9.
11. Crisa L, Cirulli V, Ellisman MH, Ishii JK, Elices MJ, Salomon DR. Cell adhesion and migration are regulated at distinct stages of thymic T cell development: the roles of fibronectin, VLA 4, and VLA 5. J Exp Med 1996;184:215-28.
12. Sawada M, Nagamine J, Takeda K, Utsumi K, Kosugi A, Tatsumi Y, et al. Expression of VLA-4 on thymocytes. Maturation stage-associated transition and its correlation with their capacity to adhere to thymic stromal cells. J Immunol 1992;149:3517-24.
13. Chang AC, Salomon DR, Wadsworth S, Hong MJ, Mojcik CF, Otto S, et al. α3 β1 and α6 β1 integrins mediate laminin/merosin binding and function as costimulatory molecules for human thymocyte proliferation. J Immunol 1995; 154:500-10.
14. Amarante-Mendes GP, Chammas R, Abrahamson P, Patel PC, Potworowski EF, Macedo MS. Cloning of a thymic stromal cell capable of protecting thymocytes from apoptosis. Cell Immunol 1995;161:173-80.
15. Moon Gyo K, Gwanghee L, Suk-Keun L, Martijn L, Jeongbin Y, Seung HH, et al. Epithelial cell-specific laminin 5 is required for survival of early thymocytes. J Immunol 2000; 165:192-201.
16. Ma F, Manabe A, Wang D, Ito M, Kikuchi A, Wada M, et al. Growth of human T cell acute lymphoblastic leukemia lymphoblasts in NOD/SCID mouse fetal thymus organ culture. Leukemia 2002;16:1541-54.
17. von Freeden-Jeffry U, Vieira P, Lucian LA, McNeil T, Burdach SE, Murray R. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J Exp Med 1995;181:1519-26.
18. Peschon JJ, Morrissey PJ, Grabstein KH, Ramsdell FJ, Maraskovsky E, Gliniak BC, et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. J Exp Med 1994;180:1955-60.
19. Plum J, De Smedt M, Leclercq G, Verhasselt B, Vandekerckhove B. Interleukin-7 is a critical growth factor in early human T-cell development. Blood 1996;88:4239-45.
20. Rich BE, Campos-Torres J, Tepper RI, Moreadith RW, Leder P. Cutaneous lymphoproliferation and lymphomas in interleukin 7 transgenic mice. J Exp Med 1993;117:305-16.
21. Dibirdik I, Langlie MC, Ledbetter JA, Tuel-Ahlgren L, Obuz V, Waddick KG, et al. Engagement of interleukin-7 receptor stimulates tyrosine phosphorylation, phosphoinositide turnover, and clonal proliferation of human T-lineage acute lymphoblastic leukemia cells. Blood 1991;78:564-70.
22. Makrynikola V, Kabral A, Bradstock K. Effects of interleukin 7 on the growth of clonogenic cells in T-cell acute lymphoblastic leukaemia. Leuk Res 1991;15:879-82.
23. Masuda M, Takanashi M, Motoji T, Oshimi K, Mizoguchi H. Effects of interleukins 1-7 on the proliferation of T-lineage acute lymphoblastic leukemia cells. Leuk Res 1991; 15: 1091-6.
24. Consolini R, Legitimo A, Cattani M, Simi P, Mattii L, Petrini M, et al. The effect of cytokines, including IL4, IL7, stem cell factor, insulin-like growth factor on childhood acute lymphoblastic leukemia. Leuk Res 1997;21:753-61.
25. Duyn AE, Kaspers GJ, Pieters R, Van Zantwijk CH, Broekema GJ, Hahlen K, et al. Effects of interleukin 3, interleukin 7, and B-cell growth factor on proliferation and drug resistance in vitro in childhood acute lymphoblastic leukemia. Ann Hematol 1999;78:163-71.
26. Karawajew L, Ruppert V, Wuchter C, Kosser A, Schrappe M, Dorken B, et al. Inhibition of in vitro spontaneous apoptosis by IL-7 correlates with bcl-2 up-regulation, cortical/mature immunophenotype, and better early cytoreduction of childhood T-cell acute lymphoblastic leukemia. Blood 2000; 96: 297-306.
27. Barata JT, Cardoso AA, Nadler LM, Boussiotis VA. Interleukin-7 promotes survival and cell cycle progression of T-cell acute lymphoblastic leukemia cells by down-regulating the cyclin-dependent kinase inhibitor p27(kip1). Blood 2001;98:1524-31.
28. Green H, Kehinde O, Thomas J. Growth of cultured human epidermal cells into multiple epithelia suitable for grafting. Proc Natl Acad Sci USA 1979;76:5665-8.
29. Ramarli D, Scupoli MT, Fiorini E, Poffe O, Brentegani M, Villa A, et al. Thymocyte contact or mAb-mediated clustering of α3β1 or α6β4 integrins activate IL-6 transcription factors (NF-κB and NF-IL6) and IL-6 production in human thymic epithelial cells. Blood 1998;92:3745-55.
30. Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 1975;6:331-43.
31. Basso G, Buldini B, De Zen L, Orfao A. New methodologic approaches for immunophenotyping acute leukemias. Haematologica 2001;86:675-92.
32. Scupoli MT, Fiorini E, Marchisio PC, Poffe O, Tagliabue E, Brentegani M, et al. Lymphoid adhesion promotes human thymic epithelial cell survival via NF-kB activation. J Cell Sci 2000;113:169-77.
33. Goodwin RG, Lupton S, Schmierer A, Hjerrild KJ, Jerzy R, Clevenger W, et al. Human interleukin 7: molecular cloning and growth factor activity on human and murine B-lineage cells. Proc Natl Acad Sci USA 1989;86:302-6.
34. Hare KJ, Jenkinson EJ, Anderson G. An essential role for the IL-7 receptor during intrathymic expansion of the positively selected neonatal T cell repertoire. J Immunol 2000;165: 2410-4.
35. Braga VM. Cell-cell adhesion and signalling. Curr Opin Cell Biol 2002;14:546-56.
36. Damsky CH, Ilic D. Integrin signaling: it's where the action is. Curr Opin Cell Biol 2002;14:594-602.
37. Winter SS, Sweatman JJ, Lawrence MB, Rhoades TH, Hart AL, Larson RS. Enhanced T-lineage acute lymphoblastic leukaemia cell survival on bone marrow stroma requires involvement of LFA-1 and ICAM-1. Br J Haematol 2001; 115:862-71.
38. Andijelic S, Jain N, Nikolic-Zugic J. Immature thymocytes become sensitive to calcium-mediated apoptosis with the onset of CD4, CD8 and the T cell receptor expression: a role for bcl-2? J Exp Med 1993;178:1745-51.
39. Scollay R. T-cell subset relationships in thymocyte development. Curr Opin Immunol. 1991;3:204-9.
40. Akashi K, Kondo M, Weissman IL. Role of interleukin-7 in T-cell development from hematopoietic stem cells. Immunol Rev 1998;165:13-28.
41. von Freeden-Jeffry U, Solvason N, Howard M, Murray R. The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression. Immunity 1997;7:147-54.
42. Schluns KS, Kieper WC, Jameson SC, Lefrancois L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat Immunol 2000;1:426-32.
43. Geginat J, Sallusto F, Lanzavecchia A. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4+ T cells. J Exp Med 2001;194: 1711-9.
44. Uckun FM, Tuel-Ahlgren L, Obuz V, Smith R, Dibirdik I, Hanson M, et al. Interleukin 7 receptor engagement stimulates tyrosine phosphorylation, inositol phospholipid turnover, proliferation, and selective differentiation to the CD4 lineage by human fetal thymocyte. Proc Natl Acad Sci USA 1991; 88: 6323-7.
45. Pallard C, Stegmann AP, van Kleffens T, Smart F, Venkitaraman A, Spits H. Distinct roles of the phosphatidylinositol 3-kinase and STAT5 pathways in IL-7-mediated development of human thymocyte precursors. Immunity 1999;10:525-35.
46. Hofmeister R, Khaled AR, Benbernou N, Rajnavolgyi E, Muegge K, Durum SK. Interleukin-7: physiological roles and mechanisms of action. Cytokine Growth Factor Rev 1999; 10:41-60.

## Pre-publication Report & Outcomes of Peer Review

*Contributions*

MTS designed, supervised the study and interpreted the results. CV and FZ contributed to carrying out experiments and to the analysis and interpretation of data. FV, MK, GN, MAR, EE and FS contributed to the analysis and interpretation of results. GP designed and supervised the study. All authors critically revised the manuscript and agreed to its final version.

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*Disclosures*

Conflict of interest: none

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*Manuscript processing*

This manuscript was peer-reviewed by two external referees and by Dr. Isabella Screpanti, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Screpanti and the Editors. Manuscript received May 7, 2003; accepted September 25, 2003.

In the following paragraphs, Dr. Screpanti summarizes the peer-review process and its outcomes.

*What is already known on this topic*

T-ALL accounts for 10-15% of pediatric and 25% of adult ALL cases and a key role of the thymus in its pathogenesis has been suggested. Indeed, the heterogeneous T-ALL subtypes are thought to correlate with aberrations at different stages of thymocyte differentiation and it has been recently shown that T-ALL blasts share the expression of specific markers with immature thymocytes. IL-7 has a critical, non-redundant role in the early stages of intrathymic T-cell differentiation and it has been shown that it specifically induces survival and proliferation of T-ALL blasts.

*What this study adds*

This is an interesting and well conducted study that certainly may add new insights to the knowledge of T-ALL pathogenesis. The finding that thymic epithelial cells produce IL-7 is new, as is the subsequent suggestion that the thymic microenvironment may have a functional role in sustaining the selective growth advantage of T-ALL blasts.

*Caveats*

This study nicely supports the functional role of the thymus in sustaining the growth of leukemic blasts. However, it is important to note that, once the leukemic cells are established and grown in the thymus, possibly following aberrations of the differentiation process, we cannot exclude that bone marrow and/or peripheral lymphoid organs, through production of other growth factors and/or integrin-mediated cell-cell interactions, may contribute to the progression and maintenance of the disease.