Cellular Oncology 32 (2010) 101–108
DOI 10.3233/CLO-2009-0500
IOS Press

Pre T-cell receptor alpha (pTα) expression patterns and functional analysis in human T-cell lymphoblastic leukemia

Philipp Ivanyi [a], Michael Morgan [a], Wenji Piao [a], Sya N. Ukena [a], Klaus Steube [b], Arnold Ganser [a] and Anke Franzke [a,*]

[a] *Clinic of Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Medizinische Hochschule Hannover, Hannover, Germany*

[b] *DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany*

Abstract. *Background*: The pTα/preTCR regulates the β-selection, a crucial T-cell developmental checkpoint, providing a most potent survival advantage to thymocytes mediated by the src-kinase p56^{Lck}.

Methods: To define the relevance of pTα in human T-cell lymphoblastic leukemia (T-ALL), we analyzed in T-ALL cell lines ($n = 14$) pTα and p56^{Lck} mRNA and protein expression as also the tyrosine-phosphorylation. The p56^{Lck} specific src-protein-tyrosine kinase inhibitor (PTK-I) PP1 was used in growth inhibition assays. IC_{50} value determination, cell cycle- and apoptosis analyses were performed in T-ALL-, non-T-ALL- and murine transgenic cell lines.

Results: pTα expression patterns were markedly different in T-ALL cell lines as compared to those reported for normal lymphoid counterparts. PP1 induced in 6/11 T-ALL cell lines a survival disadvantage resulting from a cell cycle arrest in the $G_{1/0}$ phase in thymic lymphoblastic cells and apoptosis induction in the immature cell line HSB-2, respectively. PP1 sensitive cell lines expressed the target protein p56^{Lck} and showed a corresponding P-Tyr signal.

Conclusion: Sensitivity of thymic T-ALLs to PP1 clearly underlines the impact of pTα mediated proliferation in this leukemic sub-type. In addition, p56^{Lck} represents also independently of pTα a promising therapeutical target for the src-kinase inhibitors in neoplastic lymphoid diseases.

Keywords: T-ALL, pTα, PP1, molecular targeted therapy, tyrosine kinase inhibitor

Abbreviations

IC_{50}	Cytotoxic half maximal inhibitory concentration;
PP1	4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d] pyrimidine;
PTK(-I)	protein-tyrosine kinase (inhibitor).

1. Background

Emergence of T-ALL is thought to result from specific interference of T-cell oncogenes with developmental networks regulating proliferation, survival and differentiation [10]. However, T-ALL clones retain characteristics of lymphoid precursors as illustrated by a shared expression pattern of surface molecules [4].

The β-selection, mainly controlled by pTα/preTCR, is crucial in TCRα/β^+ T-cell lineage commitment and development [23]. Before the onset of positive and negative selection, the pTα/preTCR rescues thymocytes with functional TCRβ-chain expression from apoptosis [13,23,26]. The preTCR, consisting of pTα/TCRβ-heterodimer and CD3 molecules, induces a ligand independent survival signal as well as cell cycle progression, dominantly mediated by the src-protein tyrosine kinase (PTK) p56^{Lck}, ultimately causing a unique proliferative burst [9,22,23]. This pivotal preTCR function mainly depends on pTα. Recently, a TCRβ-independent self-oligomerization of pTα was postulated for initiation of the preTCR-signal [26].

*Corresponding author: Anke Franzke, Clinic of Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Medizinische Hochschule Hannover, OE6860, Carl-Neuberg Str. 1, 30625 Hannover, Germany. Tel.: +49 511 532 3202; Fax: +49 511 532 8205; E-mail: Franzke.Anke@MH-Hannover.de.

While the relevance of pTα/preTCR expression in leukemogenesis is currently a disputed topic, leukemogenesis in Notch3-ICN transgene mice showed a striking dependence on pTα expression [1,3,7,20,27]. Additionally, the interpretation of the functional relevance of pTα/preTCR in human T-ALL cells is controversial due to the lack of antibodies, low surface expression and novel insights that implicate pTα itself, independently of other components of the preTCR as the mediator of this early survival signal [26].

Here, we analyzed pTα gene- and protein-expression patterns and employed the src-PTK-I PP1 to study the biological relevance of the pTα/preTCR in human T-ALL cell lines.

2. Materials and methods

Cell lines ($n = 18$) were obtained from the DSMZ (Braunschweig, Germany, http://www.dsmz.de). T-ALL cell lines ($n = 14$) were classified according to the EGIL-criteria [4], while pre- and pro-T-ALL due to the low number of available cell lines were categorized as immature T-ALL. Murine SCB.29 and SCBγ/δ.28 were provided by H. von Boehmer (Dana-Faber Cancer Institute, Boston, MA, USA) [17].

$pT\alpha^a$ (wild-type) expression was quantified by real-time RT-qPCR and normalized to β-actin. RT-PCR was performed for: $pT\alpha^b$ (truncated isoform), β-actin and $p56^{Lck}$ PCR conditions and primer sequences can be obtained from the corresponding author.

Western blot analysis of the pTα-chain (murine monoclonal antibody 2F5, Hybridoma J2.3, obtained by H. von Boehmer, Boston, MA, USA) and actin (murine monoclonal antibody, C2, Santa Cruz Biotechnology, CA, USA) was accomplished according to standard protocols, using equal protein lysate amounts (3×10^5 cells; $n = 20$; each sample was tested at least twice) [16]. Best signals were used for further normalization to the actin signal and semi-quantification of pTα protein expression was performed using scion image beta (V. 4.02, USA). Prior to analysis of the cell lines, the specificity of the antibody 2F5 was validated in a competitive antigen assay using crystallized pTα antigen (kindly provided by M. Probst-Kepper, Helmholtz-Center for Infection Research, Braunschweig, Germany) and β_2-Microglobulin (Santa Cruz Biotechnology, CA, USA). $p56^{Lck}$ protein (rabbit monoclonal antibody, 73A5, Cell Signaling, Boston MA, USA) and total cellular tyrosine phosphorylation (murine monoclonal antibody, P-Tyr-100, Cell Signaling, USA) were analyzed in equal cell lysate amounts (3×10^5 cells; testing the effect of PP1: 1.5×10^5 cells) by Western blotting according to standard protocols ($n = 15$; analyzed at least twice) [12,16]. β-actin was used as a loading control (monoclonal mouse antibody, AC-47, Sigma, Germany). The bound antibodies were detected by horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence (Santa Cruz Biotechnology, CA, USA and Cell Signaling, USA). Summarizing heat maps were compiled for quantitative $pT\alpha^a$-, semi-quantitative $pT\alpha^b$- and $p56^{Lck}$-mRNA and pTα protein expression with Genesis (V. 1.7.2, A. Sturn, Graz University of Technology, Austria) [19].

IC_{50} values were determined by MTT assays ($n = 16$; average was obtained from five independent experiments, each performed in triplicates; 5 mg/ml, 6 h incubation; Sigma, Germany) following PP1 incubation (96 h; tested PP1 concentrations: 0.1, 1, 10 and 20 μM; Biomol, USA). Cell cycle analyses were performed by acridine-orange (http://www.healthcare.uiowa.edu) based DNA quantification ($n = 10$ cell lines, 3 independent experiments, each as duplicate) (Sigma, Germany). Cells (8×10^5 cells) were cultured 12 h in serum-deprived medium (RPMI-1640 medium containing no FCS) and then subsequently incubated (48 h) in accordant RPMI medium prior to cell cycle staining (PP1 treated sample: 8 μM PP1 + 10% FCS; starvation control: 0.02% DMSO + 1% FCS; growth control: 0.02% DMSO + 10% FCS). Controls enabled clarification of $SubG_0$-phase- and $G_{1/0}$-phase populations. Estimated S-phase- and $G_{2/M}$ populations were summarized as the proliferative fraction of gated cells using doublet discrimination mode. From the same samples apoptotic bodies were visualized with Hoechst33258 (Sigma, Germany), and the relative numbers of vital cells out of 100 counted cells were calculated. Apoptosis was further validated and quantified by flow cytometry using an annexin-V/propidium iodide staining kit, following the manufacturer's instructions (two separate experiments; Roche Applied Science, Germany).

3. Results and conclusion

Analysis of peripheral blood mononuclear cells (PBMCs) from T-ALL patients at disease onset and in remission identified combined expression of $pT\alpha^a$, its isoform $pT\alpha^b$ and Notch3 of particular impor-

tance for sustaining T-cell leukemogenesis, potentially representing a pathognomonic molecular signature of human T-ALL [3]. In contrast to this finding but in agreement with Asnafi et al. [1], we detected mRNA expression of pTα isoforms in patients with T-ALL, B-ALL, AML and also in healthy controls (data not shown). Expression analysis of pTα in PBMCs from T-ALL patients might be misleading since pTα expression in these specimens is most likely not originating from the leukemic population, but rather from $CD4^+$ $CD3^-$ extrathymic T-cell progenitors [6], whose characteristics in T-ALL are unknown. To avoid this bias, our analyses were performed in homogenous T-ALL cell lines.

Among all analyzed T-ALL subtypes, immature T-ALLs showed the highest average $pT\alpha^a$ mRNA expression, and $pT\alpha^b$ mRNA was found in two out of three immature T-ALLs (Fig. 1A, B). pTα protein was detected in three out of three immature T-ALLs (Fig. 1D). Intermediate $pT\alpha^a$ mRNA expression was found in thymic T-ALLs, and $pT\alpha^b$ was detected in most of these cells (Fig. 1A, B). pTα protein was detected in six out of six thymic T-ALLs (Fig. 1D). Mature T-ALLs expressed the lowest level of $pT\alpha^a$ mRNA, and only two out of four mature T-ALLs expressed $pT\alpha^b$ (Fig. 1A, B), whereas all mature T-ALLs expressed pTα protein (Fig. 1D).

Physiological pTα isoform expression is differentially regulated during T-cell maturation [2,15]. Normally, $pT\alpha^a$ expression increases until β-selection onset, while the transcriptional onset of $pT\alpha^b$ is delayed, peaking after the β-selection and potentially regulating a rapid loss of $pT\alpha^a$ after β-selection, thus terminating the cellular and molecular effects of pTα [2,15]. During the thymic window, where the β-selection is supposed to occur, $pT\alpha^b$ is believed to strengthen the $pT\alpha^a$ signal [2,15]. Quantitative and qualitative differences were observed by comparing pTα mRNA expression patterns in T-ALLs with expression patterns known from thymic T-cell development. In contrast to thymocytes, maximum $pT\alpha^a$ expression was found prior to the β-selection in immature T-ALLs [2,15]. Furthermore, $pT\alpha^a$ was still expressed in mature T-ALLs, while $pT\alpha^b$ was rarely detected. The variation of $pT\alpha^a$ gene expression level throughout the immunophenotypical compartments is impressively higher among T-ALL subtypes (approximately 10.000-fold) than during intrathymic differentiation (approximately 80-fold) [15].

Even though $pT\alpha^a$ protein expression has been rarely reported in thymocyte populations corresponding to immunophenotypical immature T-ALL cells, we clearly demonstrated pTα protein translation in immature T-ALL cells (Fig. 1D). Our finding is supported by Gounari et al. [11] who observed human CD25 protein expression in murine lymphoid restricted progenitors, controlled by the pTα promoter. Since $pT\alpha^a$ expres-

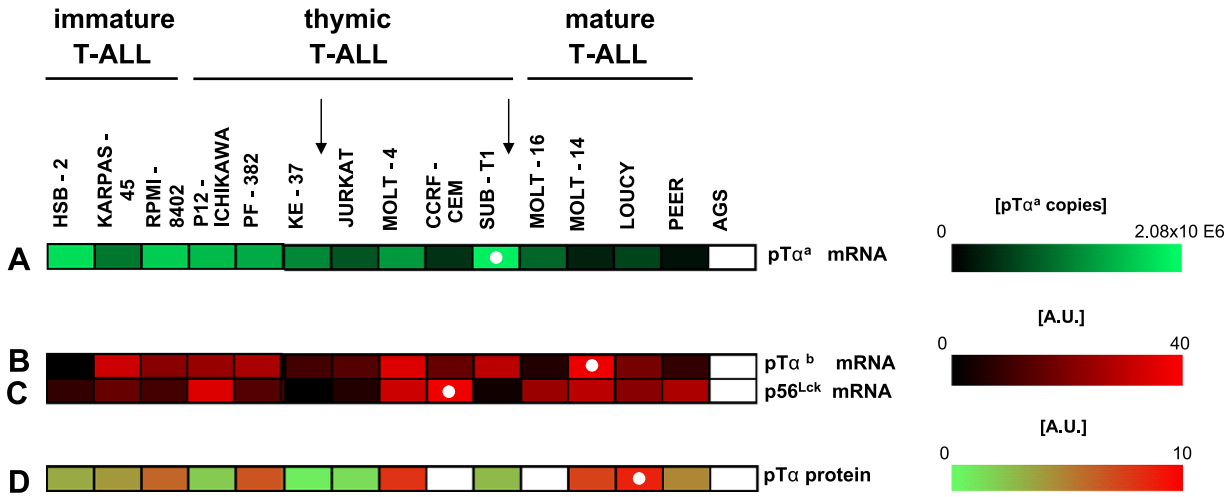

Fig. 1. Expression patterns in T-ALL cell lines for $pT\alpha^a$-, $pT\alpha^b$-, $p56^{Lck}$-mRNA and pTα protein. Heat map illustrations are shown with respect to the maturational stage of the particular leukemia cell line. (A) β-actin normalized $pT\alpha^a$ (wildtype) mRNA expression analyzed by RT-qPCR in human T-ALL cell lines (absolute quantification in copy numbers of $pT\alpha^a$). (B) β-actin normalized $pT\alpha^b$ (truncated isoform) and (C) $p56^{Lck}$ mRNA expression by RT-PCR (relative quantification in arbitrary units; A.U.). (D) Actin normalized pTα protein expression analyzed by Western blotting (relative quantification in arbitrary units; A.U.). Cell lines with the highest expression are marked by white circles. White squares indicate negative control (AGS – gastric cancer cell line), or cell lines in which protein analysis was not performed (e.g., CCRF-CEM and MOLT-16). Arrows indicate the developmental stage with expected pTα function.

sion and function is physiologically down-regulated by pTα^b in mature T-cells [2,15], it was surprising to observe pTα protein expression in mature T-ALLs. This finding correlates with unexpectedly persistent pTα^a mRNA expression concomitant with lower than expected pTα^b expression in mature T-ALLs (Fig. 1A, B) [15], suggesting an aberrantly maintained pTα signal due to a lack of physiological pTα down-regulation in mature T-ALLs. Importantly, the regulation of pTα mRNA-isoforms and pTα protein expression is unknown. Here, we interestingly observed a tendency of mRNA/protein proportion from high/low in immature T-ALLs inverting during further maturated phenotypes to low/high in mature T-ALLs (Fig. 1A, D). Whereas the known function of T-cell receptors is in general linked to defined stages, cellular environment and pTα/preTCR signal intensity, one might speculate that the mRNA/protein expression profile in the thymic window reflects the physiological differentiation; the expression profile in immature and mature T-ALL differs strikingly from this physiological situation, possibly presenting an abnormal and/or additional regulation of pTα gene/protein expression.

To better define the functional relevance of pTα in T-ALL cell lines, we used the src-PTK-I PP1 to block proximal pTα/preTCR signaling, which is mainly mediated through p56^{Lck} [22,23]. Initially, p56^{Lck} mRNA (Fig. 1C) and protein expression as well as tyrosine-phosphorylation were analyzed (Fig. 2). The immature T-ALLs showed low levels of p56^{Lck} mRNA (Fig. 1C). On the protein level, HSB-2 showed strong p56^{Lck} and P-Tyr signals (Fig. 2C), whereas a second immature T-ALL cell line, RPMI-8402, exhibited lower p56^{Lck} and total phosphorylated protein levels. All thymic T-ALLs

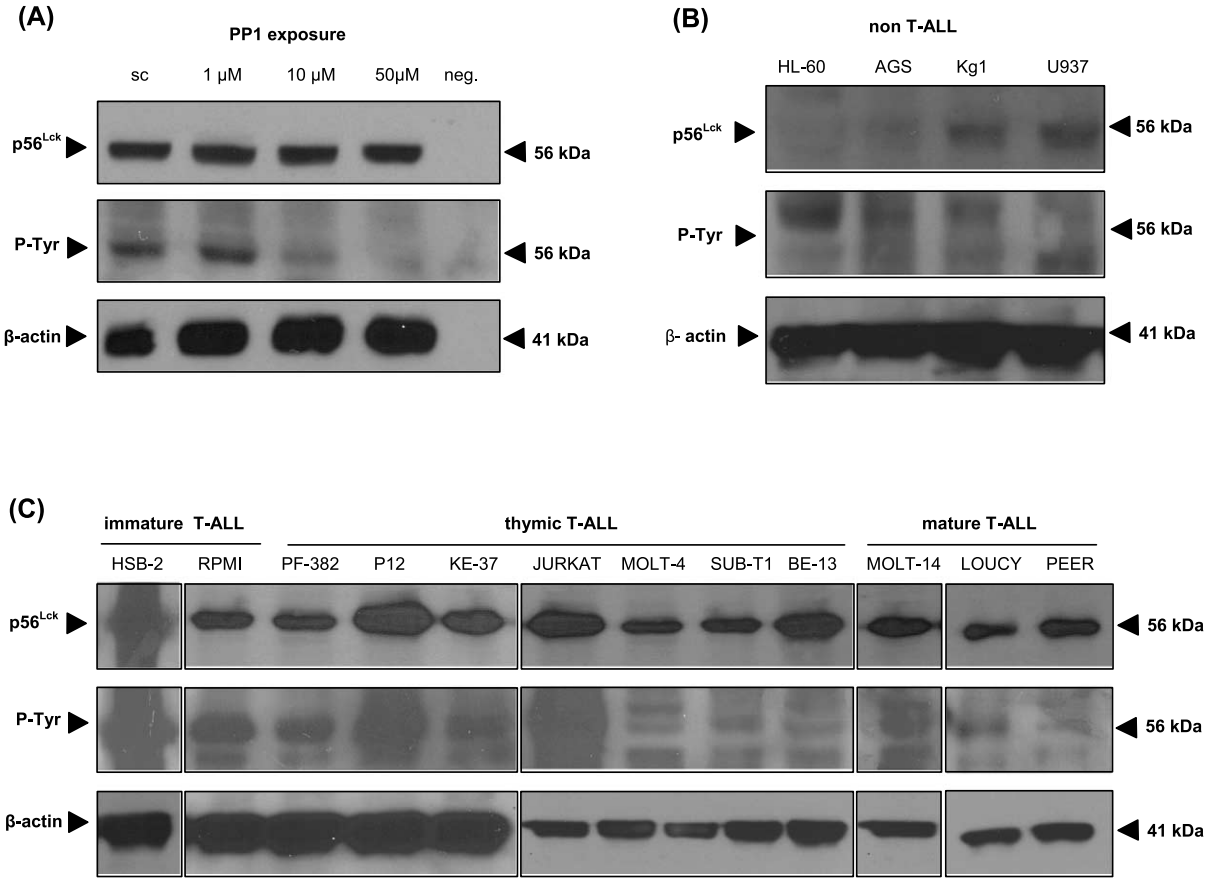

Fig. 2. Western blot analysis of p56^{Lck} protein expression, total cellular tyrosine phosphorylated proteins (P-Tyr) and β-actin in human leukemic cell lines. (A) The human T-ALL cell line JURKAT (1×10^6 cells) was incubated either with the control (0.02% DMSO) or with PP1 at the respective concentrations (neg. – no protein control). After 48 h incubation equal cell lysate amounts (1.5×10^5 cells) were analyzed for p56^{Lck} protein expression (56 kDa), P-Tyr status and β-actin (41 kDa) by Western blotting as described in Section 2. (B, C) Equal cell lysate amounts (3×10^5 cells) of human non-T-ALL cell lines (B) and human T-ALL cell lines (C) were analyzed for the expression of p56^{Lck} (56 kDa), tyrosine phosphorylated proteins and β-actin (41 kDa). Abbreviations: sc = solvent control, RPMI = RPMI-8402, P12 = P12-ICHIKAWA.

showed $p56^{Lck}$ mRNA, protein expression and a corresponding P-Tyr signal. In the mature T-ALLs, strong $p56^{Lck}$ mRNA and protein expression were detected with a lower corresponding P-Tyr signal (Fig. 2C).

PP1 is a highly potent and specific inhibitor of the src-kinase family, mainly targeting $p56^{Lck}$ [12]. Cells depending essentially on the pTα/preTCR signal were expected to have survival disadvantages at lower PP1 concentrations compared to cells which are independent of pTα/preTCR signaling. As previously shown, PP1 reduced $p56^{Lck}$ tyrosine-phosphorylation in a dose-dependent manner (Fig. 2A) [12]. Furthermore, we tested PP1 activity in MOLT-4 and HL-60 cells (Fig. 3A): $p56^{Lck}$-positive MOLT-4 cells were far more sensitive to PP1 (IC_{50}: 5.99 μM) as compared to the $p56^{Lck}$-negative AML cell line HL-60 (IC_{50}: >20 μM) (Figs 1C, 2B, C, 3A). To investigate pTα/preTCR-dependent versus pTα/preTCR-independent $p56^{Lck}$ activation, two transgenic thymocyte cell lines were used. SCB.29 cells, in which $p56^{Lck}$ activation is known to be pTα-dependent, are twice as sensitive to PP1 in comparison to SCBγ/δ.28 cells, in which pTα/preTCR has no relevance (IC_{50}: 6.9 μM vs. 13.99 μM, respectively) (Fig. 3A) [17]. Therefore, an IC_{50} <10 μM PP1 was considered as specific inhibition of pTα/preTCR proximal signaling. Using these criteria, we observed functional pTα/preTCR expression in six out of eleven tested T-ALL cell lines (Fig. 3A).

The highest PP1 responsiveness (IC_{50}: 0.96 μM) was observed in HSB-2 (Fig. 3A), one of two tested immature T-ALLs; further analyses could demonstrate that PP1 exposure resulted in apoptosis induction of HSB-2 (Figs 3B, 4A, B), clearly shown by nearly 90% annexin-V positive cells. In four out of six thymic T-ALL cell lines PP1 induced a $G_{1/0}$ arrest and caused moderate growth inhibition in one out of three mature T-ALL cell lines, without any evidence of apoptosis (Figs 3B, 4). All PP1 sensitive cell lines expressed the target protein $p56^{Lck}$ and showed a corresponding P-Tyr signal (Fig. 2C). Two out of six thymic T-ALLs, which are early thymic T-ALLs according to the immunophenotype, did not show PP1 sensitivity, even though they expressed $p56^{Lck}$ with corresponding P-Tyr signals (Fig. 2C). One might speculate, that in these PP1 non-sensitive thymic T-ALL cell lines alternative oncogenic pathways independent of phosphory-

(A)

subtype	cell line	IC_{50} [μM]
immature T-ALL	HSB-2	0.96
	RPMI-8402	14.66
thymic T-ALL	P12-ICHIKAWA	15.22
	PF-382	13
	KE-37	7.65
	JURKAT	7.76
	MOLT-4	5.99
	SUB-T1	8.75
mature T-ALL	MOLT-14	9.41
	PEER	20
	LOUCY	>20
AML	HL-60	>20
	U-937	17.33
B-ALL	TANOUE	15.71
murine	SCB.29	6.9
	SCBγ/δ.28	13.99

(B)

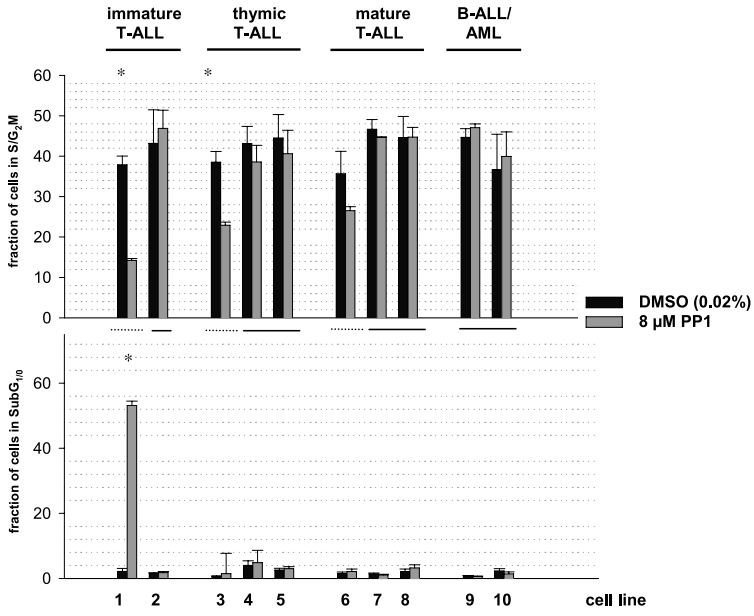

Fig. 3. Functional analysis in transgenic cell lines and T-ALL cell lines. (A) IC_{50} values in human cell lines (T-ALL and non-T-ALL) after PP1 exposure; mean IC_{50} value of 5 experiments is shown – each experiment was performed as triplicate. (B) Cell cycle analysis of PP1 sensitive (<10 μM PP1; dotted line) and non-sensitive cell lines (continuous line), previously defined by IC_{50} determination. Results for PP1 treated cell lines and controls (0.02% DMSO) are shown; average of 2 experiments is shown – each experiment was performed as triplicate. (*) $p \leqslant 0.01$ for PP1 (8 μM) treated cells compared to control. (1) HSB-2, (2) RPMI-8402, (3) MOLT-4, (4) P12-ICHIKAWA, (5) PF-382, (6) MOLT-14, (7) LOUCY, (8) PEER, (9) TANOUE, (10) HL-60.

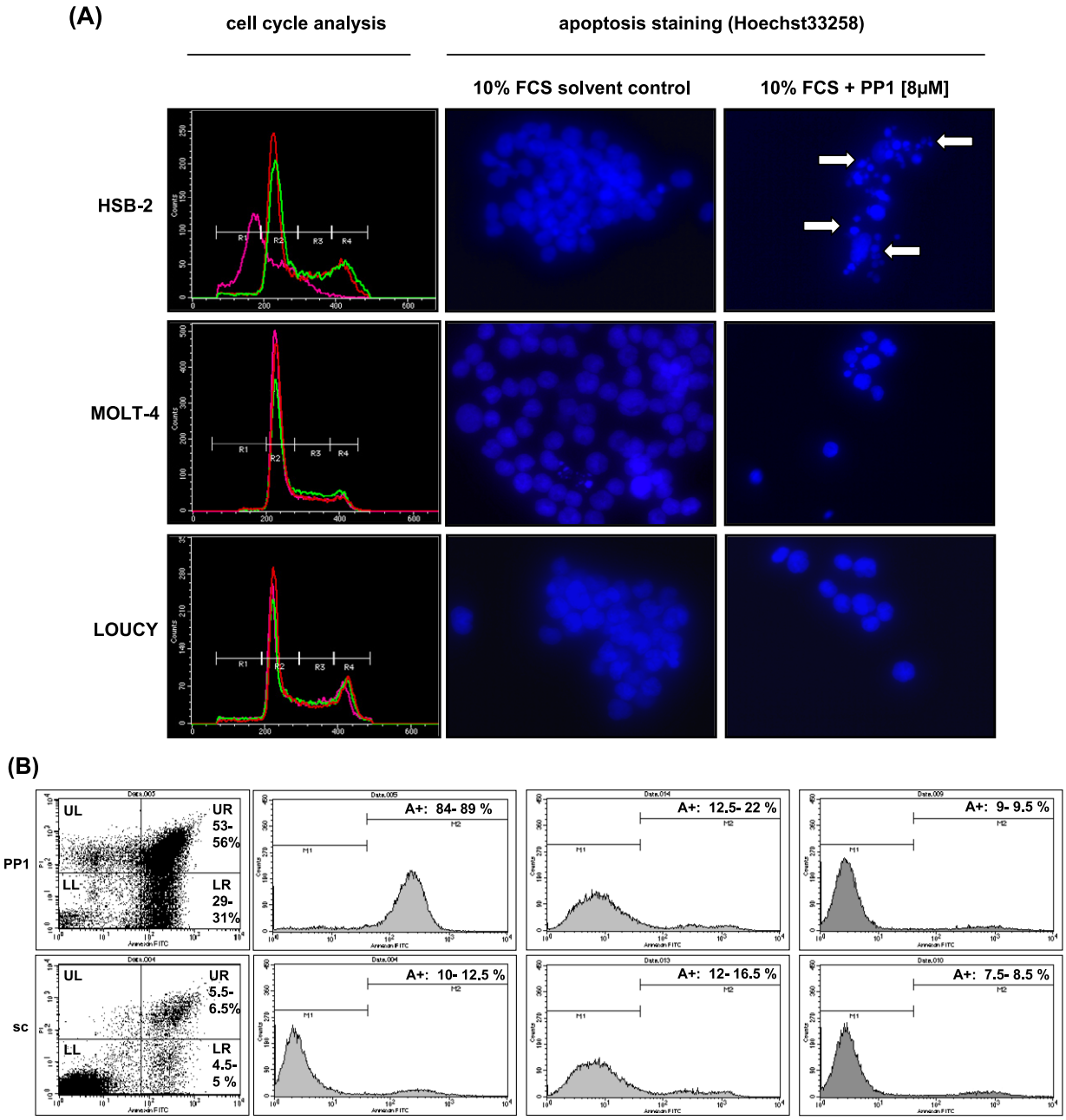

Fig. 4. Analysis of the growth inhibitory mechanism after PP1 treatment in human T-ALL cell lines. (A) Cell cycle analysis and apoptosis staining after PP1 exposure with Hoechst33258 performed with the cell lines HSB-2 (highly PP1 sensitive), MOLT-4 (PP1 sensitive) and LOUCY (PP1 insensitive). Cells cycle analyses were performed five times in triplicates as described in Section 2 (R1 – $SubG_{1/0}$-fraction, R2 – $G_{1/0}$-phase fraction, R3 – S-phase fraction, R4 – G_2/M-phase fraction; red – starvation control; green – proliferative control, purple – PP1 treated cells; white arrow indicates apoptotic bodies). Summary of cell cycle analysis is shown in Fig. 3B. (B) Apoptosis was further determined by flow cytometry analysis of annexin-V/propidium iodid (PI) stained cells as described in Section 2. Representative FACS analyses are shown for the T-ALL cell lines HSB-2 (PP1 highly sensitive), MOLT-4 (PP1 sensitive), LOUCY (PP1 insensitive). Results from two independent experiments are given as range of the relative cell distribution in the dot-plots and histograms, respectively. Legend: Dot-plots: y-axis: PI, x-axis: annexin-V; lower left quadrant (LL) – vital cells, lower right quadrant (LR) – early apoptosis, upper right quadrant (UR) – late apoptosis, upper left quadrant (UL) – detritus; histograms: y-axis: event counts, x-axis: annexin-V, M1 – annexin-V negative cells, M2/A+ – annexin-V positive cells.

lated $p56^{Lck}$ deliver a more dominant survival advantage.

We were further interested in the high sensitivity of the immature T-ALL cell line HSB-2 to PP1, as pTα signaling in immature T-ALLs is very unlikely and suspected an oncogenic pathway other than pTα/preTCR. Interestingly, the chromosomal analysis of HSB-2 cells (data not shown) revealed the translocation $t(1;7)$(q34; p34) [21,25], which causes independently of pTα a hyperactivation of $p56^{Lck}$ (Fig. 2C). Even though the incidence of the translocation $t(1;7)$(q34; p34) is considerably low at the initial diagnosis of a T-ALL, it has been speculated whether or not this translocation, or that Lck in general, is involved in later genetic events causing a transformation to a progressively more aggressive leukemia [14,21,25]. Furthermore, alterations of the chromosome 1p are frequently observed in human neoplastic disease. Approximately 10% of non-Hodgkin Lymphomas had genetic alteration at the site 1p31-36, potentially affecting Lck expression [14] and thereby represent an interesting therapeutical target for src-PTK-I as PP1.

The PP1-induced $G_{1/0}$ arrest in thymic T-ALL cell lines supports the hypothesis that pTα/preTCR sustains proliferation in the leukemic thymic compartment, in which pTα activation is physiologically relevant for $G_{1/0}$ to S-phase transition and the arrested leukemogenic clone might be supported from this potent survival mediator [5,9,23]. Interestingly, not all thymic T-ALLs respond in the defined PP1 concentration range ($<$10 μM) arguing for a very tightly defined time window of malignant arrest in which pTα is relevant: PP1 unresponsive cell lines are immunophenotypically early thymic T-ALLs, whereas the responsive cell lines are considered to be in the intermediate and late thymic developmental time window. With respect to T-cell leukemogenesis further dissection of the Notch/pTα/CyclinD3 signaling axis is needed, as Notch1 or CyclinD3 inhibition, upstream or downstream of the pTα/preTCR, also cause $G_{1/0}$ arrest predominantly in thymic T-ALLs [8,18,24].

In summary, our data point at the crucial role of pTα in defined subtypes of T-ALL mediating survival advantages for the malignant cell-type. Additionally, using the src-PTK-I PP1, we demonstrated that $p56^{Lck}$ also independently of pTα represents a promising therapeutical target in neoplastic T-cell diseases.

Acknowledgments

This work was supported by the Dieter-Schlag Stiftung, Germany. We are grateful to Ludwig Wilkens, Institute of Cell and Molecular Pathology, Medizinische Hochschule Hannover, for performing the cytogenetic analysis of the cell lines. We thank Utz Krug and Victor Grünwald for helpful technical advice and critical discussion.

References

[1] V. Asnafi, K. Beldjord, E. Boulanger, B. Comba, P. Le Tutour, M.H. Estienne, F. Davi, J. Landman-Parker, P. Quartier, A. Buzyn, E. Delabesse, F. Valensi and E. Macintyre, Analysis of TCR, pT alpha, and RAG-1 in T-acute lymphoblastic leukemias improves understanding of early human T-lymphoid lineage commitment, *Blood* **101** (2003), 2693–2703.

[2] D.F. Barber, L. Passoni, L. Wen, L. Geng and A.C. Hayday, The expression *in vivo* of a second isoform of pT alpha: implications for the mechanism of pT alpha action, *Journal of Immunology* **161** (1998), 11–16.

[3] D. Bellavia, A.F. Campese, S. Checquolo, A. Balestri, A. Biondi, G. Cazzaniga, U. Lendahl, H.J. Fehling, A.C. Hayday, L. Frati, H. von Boehmer, A. Gulino and I. Screpanti, Combined expression of pTalpha and Notch3 in T cell leukemia identifies the requirement of preTCR for leukemogenesis, *Proceedings of the National Academy of Sciences of the United States of America* **99** (2002), 3788–3793.

[4] M.C. Bene, G. Castoldi, W. Knapp, W.D. Ludwig, E. Matutes, A. Orfao and M.B. van't Veer, Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL), *Leukemia* **9** (1995), 1783–1786.

[5] B. Blom, M.C. Verschuren, M.H. Heemskerk, A.Q. Bakker, E.J. van Gastel-Mol, I.L. Wolvers-Tettero, J.J. van Dongen and H. Spits, TCR gene rearrangements and expression of the pre-T cell receptor complex during human T-cell differentiation, *Blood* **9** (1999), 3033–3043.

[6] L. Bruno, P. Res, M. Dessing, M. Cella and H. Spits, Identification of a committed T cell precursor population in adult human peripheral blood, *Journal of Experimental Medicine* **185** (1997), 875–884.

[7] A.F. Campese, A.I. Garbe, F. Zhang, F. Grassi, I. Screpanti and H. von Boehmer, Notch1-dependent lymphomagenesis is assisted by but does not essentially require pre-TCR signalling, *Blood* **108** (2006), 305–310.

[8] M. Ciofani and J.C. Zúniga-Pflücker, Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism, *Nature Immunology* **9** (2005), 881–888.

[9] I. Falk, J. Biro, H. Kohler and K. Eichmann, Proliferation kinetics associated with T cell receptor-beta chain selection of fetal murine thymocytes, *Journal of Experimental Medicine* **1** (1996), 2327–2239.

[10] A.A. Ferrando, D.S. Neuberg, J. Staunton, M.L. Joh, S.C. Raimondi, F.G. Behm, C.H. Pui, J.R. Downing, D.G. Gilliland, E.S. Lander, T.R. Golub and A.T. Look, Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia, *Cancer Cell* **1** (2002), 75–87.

[11] F. Gounari, I. Aifantis, C. Martin, H.J. Fehling, S. Hoeflinger, P. Leder, H. von Boehmer and B. Reizis, Tracing lymphopoiesis with the aid of a pTalpha-controlled reporter gene, *Nature Immunology* **3** (2002), 489–496.

[12] J.H. Hanke, J.P. Gardner, R.L. Dow, P.S. Changelian, W.H. Brissette, E.J. Weringer, B.A. Pollok and P.A. Connelly, Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-depnendent T cell activation, *Journal of Biological Chemistry* **2** (1996), 695–701.

[13] M. Mandal, C. Borowski, T. Palomero, A.A. Ferrando, P. Oberdoerffer, F. Meng, A. Ruiz-Vela, M. Cofani, J.C. Zungia-Pflucker, I. Screpanti, A.T. Look, S.J. Korsmeyer, K. Rajewsky, H. von Boehmer and I. Aifantis, The BCL2A1 gene as a pre-T cell receptor-induced regulator of thymocyte survival, *Journal of Experimental Medicine* **201** (2005), 603–614.

[14] J.D. Marth, C. Disteche, D. Pravtecheva, F. Ruddle, E.G. Krebs and R.M. Perlmutter, Localization of a lymphocyte-specific protein tyrosine kinase gene (lck) at a frequent chromosomal abnormalities in human lymphomas, *Proceedings of the National Academy of Sciences of the United States of America* **19** (1986), 7400–7404.

[15] A.R. Ramiro, M.N. Navarro, A. Carreira, Y.R. Carrasco, V.G. de Yébenes, G. Carrello, J.L. San Millán, B. Rubin and M.L. Toribio, Differential developmental regulation and functional effects on pre-TCR surface expression of human pTalpha(a) and pTalpha(b) spliced isoform, *Journal of Immunology* **167** (2001), 5106–5114.

[16] C. Saint-Ruf, O. Lechner, J. Feinberg and H. von Boehmer, Genomic structure of the human pre-TCR receptor alpha chain and expression of two mRNA isoforms, *European Journal of Immunology* **11** (1998), 2824–2831.

[17] C. Saint-Ruf, M. Panigada, O. Azogui, P. Debey, H. von Boehmer and F. Grassi, Different initiation of pre-TCR and gammadeltaTCR signalling, *Nature* **406** (2000), 524–527.

[18] E. Sicinska, I. Aifantis, L. Le Cam, W. Swat, C. Borowski, Q. Yu, A.A. Ferrando, S.D. Levin, Y. Geng, H. von Boehmer and P. Sicinski, Requirement for cyclin D3 in lymphocyte development and T cell leukemias, *Cancer Cell* **4** (2003), 451–461.

[19] A. Sturn, J. Quackenbush and Z. Trajanoski, Genesis: cluster analysis of microarray data, *Bioinformatics* **1** (2002), 207–208.

[20] C. Talora, A.F. Campese, D. Bellavia, M. Pascucci, S. Checquolo, M. Groppioni, L. Frati, H. von Boehmer, A. Gulino and I. Screpanti, Pre-TCR-triggered ERK signalling-dependent downregulation of E2A activity in Notch3-induced T-cell lymphoma, *EMBO Reports* **4** (2003), 1067–1072.

[21] B. Tycko, S.D. Smith and J. Sklar, Chromosomal translocations joining LCK and TCRB loci in human T cell leukemia, *Journal of Experimental Medicine* **4** (1991), 867–873.

[22] N.S. van Oers, B. Lowin-Kropf, D. Finlay, K. Conolly and A. Weis, $\alpha\beta$ T cell development is abolished in mice lacking both Lck and Fyn protein tyrosine kinases, *Immunity* **5** (1996), 429–436.

[23] H. von Boehmer, Unique features of the pre-T-cell receptor alpha-chain: not just a surrogate, *Nature Reviews Immunology* **5** (2005), 571–577.

[24] A.P. Weng, A.A. Ferrando, W. Lee, J.P. Morris 4th, L.B. Silverman, C. Sanchez-Irizarry, S.C. Blacklow, A.T. Look and J.C. Aster, Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia, *Science* **5694** (2004), 269–271.

[25] D.D. Wright, B.M. Sefton and M.P. Kamps, Oncogenic activation of the Lck protein accompanies translocation of the LCK gene in the human HSB2 T-cell leukemia, *Molecular and Cellular Biology* **14** (1994), 2429–2437.

[26] S. Yamasaki, E. Ishikawa, M. Sakuma, K. Ogata, K. Sakata-Sogawa, M. Hiroshima, D.L. Wiest, M. Tokunaga and T. Saito, Mechanistic basis of pre-T cell receptor-mediated autonomous signaling critical for thymocyte development, *Nature Immunology* **7** (2006), 67–75.

[27] A. Vacca, M.P. Felli, R. Palermo, G. DiMario, A. Calce, M. Di Giovine, L. Frati, A. Gulino and I. Screpanti, Notch3 and pre-TCR interaction unveils distinct NF-kappaB pathways in T-cell development and leukemia, *EMBO Journal* **25** (2006), 1000–1008.