NOTCH1 pathway activation is an early hallmark of SCL T leukemogenesis

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The acquired activation of stem cell leukemia (SCL) during T lymphopoiesis is a common event in T-cell acute lymphoblastic leukemia (T-ALL). Here, we generated tamoxifen (TAM)–inducible transgenic mice (lck-ER^{T2}-SCL) to study the consequences of acquired SCL activation during T-cell development. Aberrant activation of SCL in thymocytes resulted in the accumulation of immature CD4$^+$CD8$^+$ (double-positive, DP) cells by preventing normal surface expression of the T-cell receptor $\alpha\beta$ (TCR$\alpha\beta$) complex. SCL-induced immature DP cells were further characterized by up-regulated NOTCH1 and generated noncycling polyclonal CD8$^+$TCRβ^{low} cells. The prevalence of these cells was SCL dependent because TAM withdrawal resulted in their disappearance. Furthermore, we observed that SCL activation led to a dramatic up-regulation of NOTCH1 target genes (Hes-1, Deltex1, and CD25) in thymocytes. Strikingly, NOTCH1 target gene up-regulation was already observed after short-term SCL induction, implying that enhanced NOTCH signaling is mediated by SCL and is not dependent on secondary genetic events. These data represent the basis for a novel pathway of SCL-induced leukemogenesis and provide a functional link between SCL and NOTCH1 during this process. (Blood. 2007;110:3753-3762)

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Introduction

The basic helix-loop-helix (bHLH) transcription factor stem cell leukemia (SCL, also known as tal1 or tcl-5) is aberrantly expressed in a high proportion of pediatric and adult cases of T-cell acute lymphoblastic leukemia (T-ALL).[1,2] Normally, SCL is expressed in hematopoietic progenitor, erythroid and megakaryocytic cells, endothelium, and the central nervous system.[3,4] Within the thymus, regular SCL expression is restricted to the most primitive cellular compartment neither expressing CD4 nor CD8, referred to as double-negative (DN) cells.[5,6] During normal T-cell development, the in-frame rearrangement of the TCRβ chain drives the differentiation of DN cells into double-positive (DP, CD4$^+$CD8$^+$) cells via an intermediate CD8 immature single-positive (ISP) stage. This process, termed β-selection, is regulated by the pre-TCR, which comprises the CD3 complex in association with the rearranged TCRβ chain and the invariant pre-TCRα chain.[7] Subsequently, the TCRα locus is recombined resulting in intermediate levels of the TCRαβ complex on the surface of DP cells. The majority of DP thymocytes die, either because they fail to express a TCR capable of interaction with self-peptide MHC complexes or because they bind too efficiently ("negative selection"). The appropriate, intermediate level of TCR signaling initiates effective maturation ("positive selection").

Ectopic expression of SCL from the DN stage onward during T lymphopoiesis is thought to be one of the initiating events of T leukemogenesis. It was shown that the aberrant expression of SCL can be caused by chromosomal translocations and intrachromosomal deletions as well as by unknown mechanisms leading to the biallelic up-regulation of SCL.[3,5] In developing T cells, SCL has been shown to operate predominantly as a transcriptional repressor by interfering with the normal activity of the bHLH transcription factors E2A and HEB.[6,8,9] However, SCL has been shown to also act as a transcriptional activator in T cells,[10] and a recent publication argued for a composite model with SCL acting as both an activator and repressor of transcription.[11]

The oncogenic potential of SCL was demonstrated in murine models.[12-14] However, the development of leukemia was infrequent and required a long latency period in most models and was dependent on the collaboration with other oncogenes.[12,14-17] The long latency period of SCL leukemia induction implies that additional genetic events are required. In fact, activating mutations within the *NOTCH1* gene have recently been identified within human T-ALL samples expressing SCL[18] and also within malignant T lymphoblasts arising in SCL transgenic mice.[19,20]

Important unanswered questions include the specific developmental stage at which acquired SCL activation perturbs T lymphopoiesis, and whether SCL affects NOTCH1 function during T-cell development. To address these, we established a conditional model of acquired SCL activation in developing thymocytes (lck-ER^{T2}-SCL). Using this model, we identified DP and CD8 single-positive (SP) thymocytes with low TCRαβ surface expression as the cellular targets of aberrant SCL activation. Unexpectedly, we observed a precursor-product relationship between these SCL-induced DP and CD8 SP thymocytes. Furthermore, our study revealed that SCL induces an activation of the NOTCH1 pathway, thereby establishing a functional link between SCL and NOTCH1 during leukemogenesis.

Materials and methods

Generation of lck-ER^{T2}-SCL transgenic mice

The cloning strategy used to generate the lck-ER^{T2}-SCL construct is described in Document S1 (available on the *Blood* website; see the

Supplemental Materials link at the top of the online article). The 7.3-kb lck-ER^{T2}-SCL DNA fragment (Figure S1A) was excised by a *Spe*I digest from plasmid pER^{T2}SCL and was used to inject fertilized FVB/N eggs (Ozgene, Bentley, WA, Australia). Transgenic animals were identified by Southern blot with a 1-kb murine SCL probe or alternatively by polymerase chain reaction (PCR) (5′-GGAAGTCCCAACTGACCCTA-3′ and 5′-ATTTTAGGGGCGCTTACCTG-3′, amplifying a 281-bp transgene-specific product).

The induction of the lck-ER^{T2}-SCL transgene was achieved by intraperitoneal injections of TAM (1 mg in 100 μL corn oil; Sigma-Aldrich, St Louis, MO) as indicated in the figure legends or by TAM administration via the feed ("TAM-feed"; 1 g TAM/kg) as previously described.[21]

All experimental protocols were approved by the bioethics committee of the district of Düsseldorf, Germany and the Princess Margaret Hospital for Children in Perth, Australia.

Real-time RT-PCR and NOTCH1 sequence analysis

RNA was isolated from thymi and cell lines with the RNeasy kit (QIAGEN, Hilden, Germany). The RNA isolation procedure included an on-column digestion step of residual genomic DNA using DNase I. Reverse transcription (RT) was carried out using Advantage RT-for-PCR Kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. Real-time polymerase chain reactions were performed with the ABI Prism 7900HT Sequence Detector (Applied Biosystems, Foster City, CA). Reactions were carried out in triplicates with target-specific assays listed in the Supplemental Methods. For quantification of each PCR result, we calculated the δCt value between the respective target gene and the housekeeping gene (GAPDH). The averaged δCt values from each individual mouse were used to perform the statistical analysis. Averaged δCt values of transgenic samples were subtracted from averaged δCt values of wild-type samples (δδCt). The $2^{-δδCt}$ value (relative quantity) was expressed as fold change.

NOTCH1 heterodimerization (HD) and PEST domain sequence analysis was carried out with primers previously published by Lin and colleagues.[19] The PCR-amplified fragments were gel purified, and the resulting sequences were compared with wild-type NOTCH1 (GenBank no. AB100603.1).

Flow cytometry and antibodies

All monoclonal antibody-fluorochrome conjugates were purchased from BD Pharmingen (San Jose, CA), except anti-CD25 (clone PC61.5; eBioscience, San Diego, CA; corresponding isotype control clone A110-1 [BD Pharmingen]). These included antibodies against CD4 (clone RM4-5), CD8 (53–6.7), CD24 (M1/69), TCRβ (H57–597; corresponding isotype control clone Ha4/8), CD44 (IM7, A95–1), Vβ2 (B20.6, R35–95), Vβ4 (KT4, A95.1), Vβ14 (14-2, R4-22), and NOTCH1 (mN1A, MOPC-31C). The staining of cells was performed as previously described.[22] Data were acquired on a FACSCalibur or on a FACSCanto (Becton Dickinson, San Jose, CA), and analysis was performed with FlowJo software (Treestar, San Carlos, CA). Dead cells were excluded by forward and side scatter in addition to propidium iodide (PI) negativity when possible. For intracellular staining, cells were washed in 0.03% saponin as previously described[23] or the BD Cytofix/Cytoperm kit was used (Becton Dickinson). Mitochondrial membrane potential was determined by incubating thymocytes with 1 μg JC-1 (Sigma-Aldrich)/mL for 15 minutes at 37°C. Apoptosis was assessed using FITC–annexin V (Becton Dickinson). For the analysis of thymocyte proliferation, mice received a single intraperitoneal injection of BrdU (1 mg per 6 g mouse weight), and BrdU-containing (1 mg/mL) drinking water was provided until organ harvest. Thymocytes were fixed and stained with APC-anti-BrdU (Becton Dickinson). NOTCH1 staining of primary TAM-SCL tumor cells was performed on thawed samples that were frozen in liquid nitrogen.

Fetal thymic organ culture

Fetal thymi were cultured on polycarbonate filters in 2 mL Iscove modified Eagle medium, 20% fetal calf serum, and 2 mM glutamine in 24-well plates. Thymocytes were harvested after 3 to 7 days in culture by gently squeezing lobes under a 1-mL syringe plunger in fluorescence-activated cell sorting (FACS) buffer, stained, and analyzed by FACS as mentioned under "Flow cytometry and antibodies." Hydroxy-tamoxifen (OH-TAM, 100-400 nM; Sigma-Aldrich) was added to the media.

Statistics

All data are presented as means plus or minus SD. We performed all analyses (except where indicated) using 2-sample *t* tests with a 2-tailed significance set at the .05 level.

Results

Conditional SCL activation induces abnormal TCRβ^{low} thymocytes

A conditional transgenic system was generated by fusing SCL to a mutated ligand-binding domain of the human estrogen receptor (ER^{T2}).[24] In preliminary experiments, we demonstrated T-cell–specific transgene expression, TAM-dependent nuclear translocation, and functionality of the ER^{T2}-SCL fusion protein within thymocytes (Figure S1). Importantly, no immunophenotypic alterations of T-cell development were observed in lck-ER^{T2}-SCL transgenic mice in the absence of TAM treatment (Figure S2). To confirm the known SCL-mediated alterations of gene expression in the lck-ER^{T2}-SCL system, we analyzed the expression of RALDH2,[10] pre-TCRα,[9] RAG2,[25] and Ephrin B1.[11] As expected, we observed up-regulation of RALDH2 and Ephrin B1 and down-regulation of pre-TCRα and RAG2 in lck-ER^{T2}-SCL transgenic compared with wild-type thymocytes (Table 1). Next, we confirmed that continuous oral TAM administration ("TAM-feed") led to T-cell leukemia in lck-ER^{T2}-SCL mice. TAM treatment for 5 to 17 months resulted in the development of T-ALL in lck-ER^{T2}-SCL mice (in the first cohort of 23 lck-ER^{T2}-SCL mice treated with TAM, 4 animals developed T-ALL, Table 2), while no T-cell malignancies were observed in untreated lck-ER^{T2}-SCL (n = 14) nor in TAM-treated wild-type mice (n = 8) during the same observation period. The malignant nature of lck-ER^{T2}-SCL T-ALLs was confirmed in secondary transplantation experiments (data not shown). We concluded that lck-ER^{T2}-SCL transgenic animals provide a functional and conditional model of SCL-induced T leukemogenesis.

Subsequently, we studied the effect of acquired SCL activation on T-cell development using lck-ER^{T2}-SCL transgenic mice. At 3 to 4 weeks of age, mice commenced consuming TAM feed, and their thymi were analyzed at different time points for the expression of CD4, CD8, and TCRβ. The most striking observation was the

Table 1. Thymocyte gene expression alterations induced by the nuclear translocation of the ER^{T2}-SCL fusion-protein

Gene	1.6 weeks of TAM		8 weeks of TAM	
	Fold change	*P*	Fold change	*P*
RALDH2	5.34	.001	4.2	.002
Pre–T-cell receptor α	−2.4	.22	−4.3	.021
RAG2	−9.1	.02	−3.6	.091
Ephrin B1	3.0	.002	2.7	.026

mRNA was isolated from thymocytes of lck-ER^{T2}-SCL (1.6 weeks of TAM feed, n = 3; 8 weeks of TAM feed, n = 3) and littermate wild-type control mice (1.6 weeks of TAM feed, n = 4; 8 weeks of TAM feed, n = 4) and used for real-time RT-PCR analysis. Fold change data represent fold increase or decrease of gene expression (mean) imposed by the lck-ER^{T2}-SCL transgene induction.

Table 2. Phenotypic analysis of lck-ER^T2^-SCL tumors and derived cell lines

| | Primary tumor | | | Derived cell line | | | | |
| | | | | | | NOTCH1 mutations | | Fold change, quantitative RT-PCR |
Animal no.	Survival on TAM, mo	Predominant phenotype	NOTCH1+ (%)	Predominant phenotype	NOTCH1+ (%)	HD	PEST	NOTCH1
151	5	DN TCRβ^low^	ND	NA	NA	NA	NA	NA
049	11	CD8 TCRβ^low^	86	CD8 TCRβ^low^	95	None	None	3.3
044	8	CD8 TCRβ^low^	61	CD8 TCRβ^low^	94	None	56676 C>CC*	6.7
065	17	DP TCRβ^high^	51	CD8 TCRβ^low^	86	None	None	4.8

Thymic tumors of tamoxifen-treated lck-ER^T2^-SCL mice and corresponding cell lines were stained with antibodies against CD4, CD8, TCRβ, and NOTCH1 and analyzed by flow cytometry. Genomic DNA was isolated from the cell lines and NOTCH1 HD and PEST domain sequence analysis was performed. mRNA of the tumor cell lines was isolated, and quantitative RT-PCR was performed for NOTCH1. Fold change data represent change compared with normal wild-type FVB/N thymocytes.

TAM indicates tamoxifen; DN, double-negative; ND, not determined; NA, not applicable; and DP, double-positive.

*Heterozygous mutation.

accumulation of CD8+TCRβ^low^ cells (Figure 1A). These cells were at first interpreted to represent CD8 ISP cells. CD8 ISP thymocytes are intermediate transitional cells between DN and DP cells. Due to their highly proliferative nature CD8 ISP cells are large, lymphoblastic cells.[26] However, CD8+TCRβ^low^ cells of TAM-treated lck-ER^T2^-SCL mice ("TAM-SCL mice") were not large (forward scatter high [FSC^high^]) like wild-type ISP CD8 cells. Rather, these cells were as small (FSC^low^) as mature CD8+TCRβ^high^ T cells

(Figure 1A). The abnormal nature of TAM-SCL CD8+TCRβ^low^ cells was further underscored by the lower expression level of surface TCRβ compared with wild-type CD8 ISP cells. Importantly, an increased proportion of TCRβ^low^ cells was also observed within DP thymocytes of TAM-SCL mice (Figure 1A). Analogous to CD8+TCRβ^low^ cells, the proportion of small cells (FSC^low^) within DP TCRβ^low^ was also increased (data not shown). A previous study had identified abnormal CD44-expressing
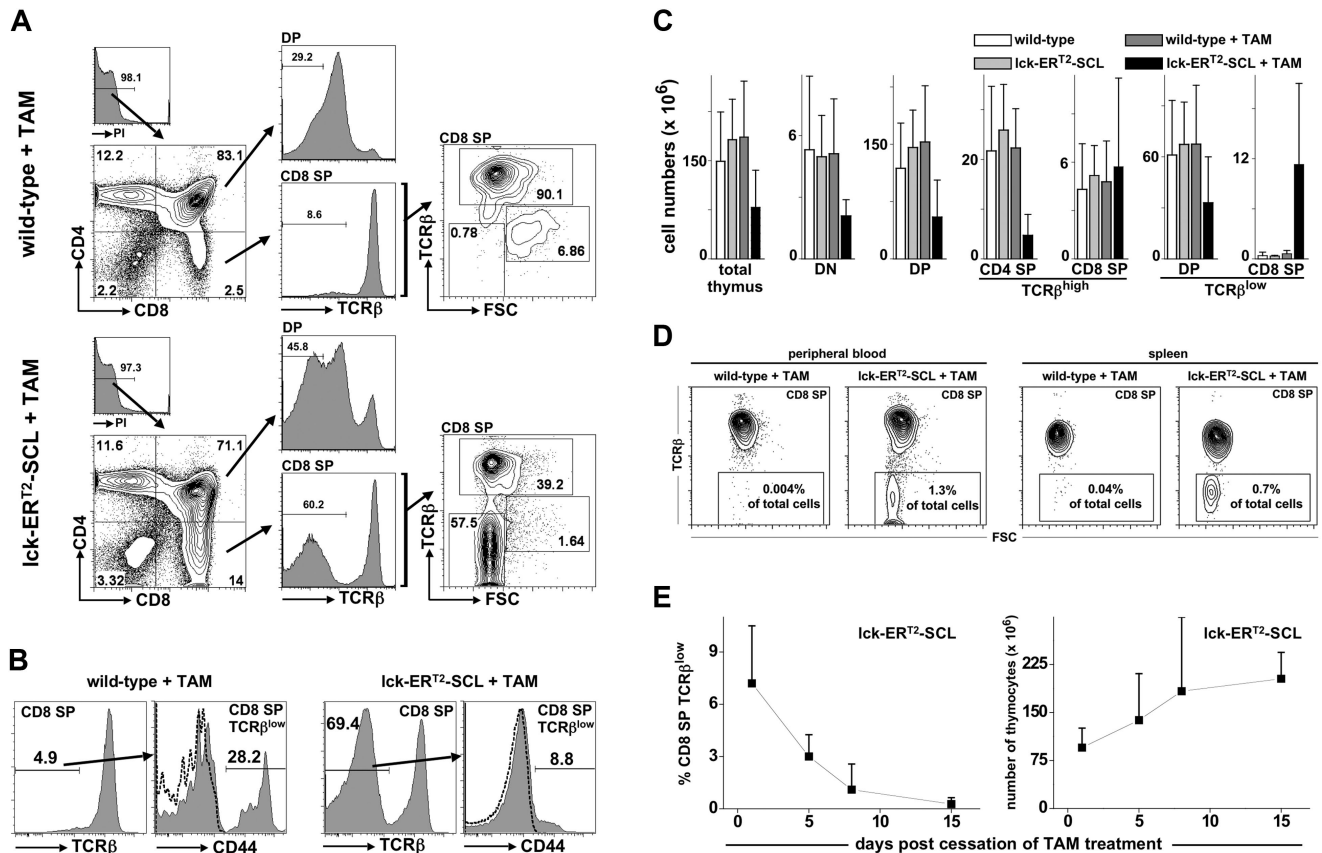

Figure 1. Conditional SCL activation induces the reversible accumulation of small CD8+TCRβ^low^ thymocytes. (A) Thymic flow cytometric analysis of tamoxifen (TAM)–treated (fed for 3 weeks) lck-ER^T2^-SCL and littermate control mice. (B) Analysis of CD44 expression on CD8+TCRβ^low^ cells. TAM feed for 6 weeks; wild-type, n = 4; lck-ER^T2^-SCL, n = 4; representative plots are shown; dashed lines represent corresponding isotype control staining. (C) Absolute cell numbers were calculated for total thymocytes and different subsets. Bars represent means plus or minus SD; wild-type no TAM, n = 10; lck-ER^T2^-SCL no TAM, n = 5; wild-type + TAM, n = 10; lck-ER^T2^-SCL + TAM, n = 13; TAM feed for 2-6 weeks. (D) CD8+TCRβ^low^ cells were detected in peripheral blood and within spleens of TAM-treated lck-ER^T2^-SCL mice. Representative plots of at least 3 mice per group are shown; TAM feed for 3 to 4 weeks. The percentage of CD8+TCRβ^low^ cells per total nucleated cells is displayed. (E) After the cessation of TAM treatment, thymic CD8+TCRβ^low^ cells disappeared and thymic cellularity returned back to normal levels. A cohort of mice (n = 13) was injected with TAM (1 mg intraperitoneally daily) for 14 days. Flow cytometric analysis of the thymus was carried out on days 1, 5, 8, and 15 after cessation of TAM administration (at least n = 3 mice per time point). The proportion of CD8+TCRβ^low^ cells per total nucleated thymocytes (left) and the total thymic cellularity (right) was calculated for each time point. Black squares represent mean (± SD). FSC indicates forward scatter; and PI, propidium iodide.

CD8$^+$TCRβ^{low} cells in SCL and LMO1 double-transgenic mice.[27] Therefore, we examined whether TAM-SCL CD8$^+$TCRβ^{low} cells also expressed CD44 (Figure 1B). In contrast to SCL and LMO1 double-transgenic mice, only a small proportion of TAM-SCL CD8$^+$TCRβ^{low} cells expressed relatively low amounts of CD44.

To further define the perturbation of thymopoiesis, we calculated absolute cell numbers of thymic subsets in TAM-SCL and control mice. In agreement with data generated with constitutive lck-SCL transgenics,[8] we observed significant reductions in total ($P < .05$), DN ($P < .001$), and DP thymocytes ($P < .001$) of TAM-SCL mice (Figure 1C) compared with TAM-treated wild-type mice. Thus, conditional activation of SCL inhibited DN generation, which subsequently reduced DP numbers. Although DP numbers were dramatically reduced in TAM-SCL thymi, normal numbers of mature CD8$^+$TCRβ^{high} were present while mature CD4$^+$TCRβ^{high} cells were dramatically decreased ($P < .001$). In contrast to all other populations analyzed, adult TAM-SCL thymi produced enhanced numbers of CD8$^+$TCRβ^{low} cells ($P < .005$) compared with control mice (Figure 1C).

Normally, T-precursor cells remain within the thymus because they are dependent on extrinsic signals uniquely provided by the thymic microenvironment.[28] Thus, we tested whether abnormal CD8$^+$TCRβ^{low} cells were capable of leaving the thymus. We were able to detect abnormal CD8$^+$TCRβ^{low} cells in peripheral blood and within spleens of TAM-SCL mice (Figure 1D). Hence, abnormal CD8$^+$TCRβ^{low} cells have the ability to leave the thymus and to survive independently of extrinsic signals inherent to the thymus.

Next, we exploited the conditional capacity of the lck-ER^{T2}-SCL model to determine whether the existence of CD8$^+$TCRβ^{low} cells is dependent on sustained SCL activation. Different lck-ER^{T2}-SCL and littermate control cohorts were treated with TAM for 14 days and their thymi were analyzed by flow cytometry 1, 5, 8, and 15 days after the cessation of TAM treatment. In addition, one lck-ER^{T2}-SCL control cohort continuously received TAM for the whole experimental period (29 days). Indeed, we observed that the appearance of abnormal CD8$^+$TCRβ^{low} cells was reversed after the cessation of TAM. Already 5 days after the cessation of TAM treatment, the proportion of abnormal CD8$^+$TCRβ^{low} cells per total thymocytes decreased by more than 50%. Fifteen days after TAM cessation, CD8$^+$TCRβ^{low} cells had completely disappeared (Figure 1E). In contrast, we detected 12.9% ($\pm$ 5.9%; mean $\pm$ SD) CD8$^+$TCRβ^{low} cells in lck-ER^{T2}-SCL thymi when TAM treatment was continued (n = 3, TAM for 29 days, data not shown). Moreover, cessation of TAM treatment caused normalization of the lck-ER^{T2}-SCL thymic cellularity (Figure 1E). These results demonstrated that sustained SCL activation was crucial for the maintenance of the abnormal CD8$^+$TCRβ^{low} cells within the thymus.

Conditional thymic SCL activation leads to the accumulation of intracellular TCRβ chains

Previous work has demonstrated that aberrant SCL expression decreases the expression of RAG2 within developing thymocytes.[8] Decreased RAG2 levels might lead to insufficient recombination at the TCRβ locus and might thus prevent TCRβ chain expression as a consequence of aberrant SCL activation. The TCRβ locus is recombined at the DN stage of thymopoiesis and the expression of the TCRβ chain is mainly restricted to the cytoplasm until the TCRα locus is recombined at the DP stage.[29] Therefore, we carried out surface and intracellular TCRβ staining in parallel: intracellular TCRβ staining should reveal whether SCL completely prevents TCRβ chain expression.

However, we were able to demonstrate that intracellular TCRβ chain expression was present in DP TCRβ^{low} and CD8$^+$TCRβ^{low} cells of TAM-SCL mice (Figure 2A,B). Interestingly, we found that TAM-SCL DP TCRβ^{low} and CD8$^+$TCRβ^{low} cells even expressed higher amounts of intracellular TCRβ chain than their wild-type control populations (Figure 2A,B). In contrast, intracellular TCRβ chain levels between mature populations (surface TCRβ^{high}) of TAM-SCL and TAM–wild-type mice did not differ significantly (Figure 2A,B). In conclusion, TAM-SCL CD8$^+$TCRβ^{low} cells expressed intracellular TCRβ chain levels that were more comparable with levels of DP thymocytes rather than wild-type CD8 ISP cells. Therefore, aberrant SCL activation did not inhibit TCRβ recombination in the majority of thymocytes. Accordingly, absent TCRβ surface expression must be due to an inability of TAM-SCL thymocytes to properly express an assembled TCRαβ complex on the cell surface.

Subsequently, we addressed by intracellular staining for different variable β (Vβ) chains whether TAM-SCL DP TCRβ^{low} and CD8$^+$TCRβ^{low} cells represent monoclonal or polyclonal populations (Figure 2C). All Vβ chains analyzed (Vβ2, Vβ4, and Vβ14) were expressed in TAM-SCL DP TCRβ^{low} and CD8$^+$TCRβ^{low} cells (Figure 2C) demonstrating that these populations were not monoclonal. Furthermore, the TAM-SCL CD8$^+$TCRβ^{low} Vβ2, 4, and 14 chain use was similar to wild-type use, further indicating that these cells were polyclonal rather than oligoclonal.

Abnormal CD8$^+$TCRβ^{low} cells are derived from CD4$^+$CD8$^+$ double-positive thymocytes

A number of reasons prompted us to hypothesize that atypical SCL-induced CD8$^+$TCRβ^{low} cells were derived from DP TCRβ^{low} rather than DN or CD8 ISP thymocytes: First, in addition to the accumulation of CD8$^+$TCRβ^{low} thymocytes, we also observed an increased proportion of TCRβ^{low} cells within DP cells. Second, in TAM-SCL thymi we detected the parallel existence of small and large CD8$^+$TCRβ^{low} cells (Figure 1A). This dichotomy was not due to transgene position effect variegation because it was also observed within other founder lines (data not shown). The small proportion of large CD8$^+$TCRβ^{low} cells might represent ISPs becoming DPs, while small CD8$^+$TCRβ^{low} cells might be abnormally derived from DPs. Third, the intracellular TCRβ chain levels of TAM-SCL CD8$^+$TCRβ^{low} cells were similar to intracellular TCRβ chain levels of DP thymocytes. In addition, we analyzed HSA (heat stable antigen, CD24) expression on SCL-induced CD8$^+$TCRβ^{low} thymocytes. Normally, HSA is progressively down-regulated during T-cell development. Remarkably, we found an intermediate HSA expression in the SCL-induced CD8$^+$TCRβ^{low} cells, which was overlapping with the HSA expression of DP TCRβ^{low} cells. However, it was dramatically lower than the HSA expression of normal wild-type CD8 ISP (CD8$^+$TCRβ^{low}) cells (Figure S3).

Therefore, we analyzed in vitro cultures of day 15.5 (E15.5) fetal lck-ER^{T2}-SCL and littermate control thymi (cultured in the presence of OH-TAM) on consecutive days to determine whether small CD8$^+$TCRβ^{low} cells appear before or after the first detection of DP thymocytes in culture. If small CD8$^+$TCRβ^{low} cells develop before the first appearance of DP cells, this would imply these cells are either derived from DN thymocytes or are abnormal CD8 ISP cells. If they are detectable after the first appearance of DP thymocytes, this argues for DP thymocytes being the precursor of TAM-SCL CD8$^+$TCRβ^{low} cells. After 3 days of culture, E15.5 fetal lck-ER^{T2}-SCL thymocytes had developed into DP thymocytes (Figure 3). In these OH-TAM–containing lck-ER^{T2}-SCL cultures,
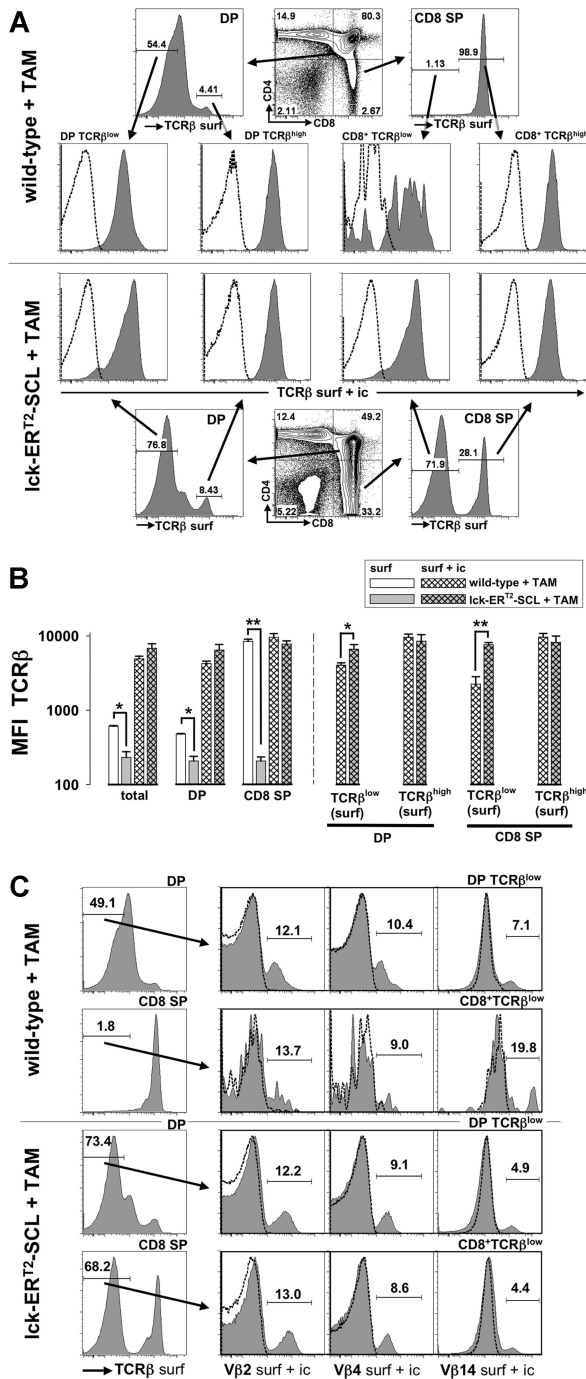
Figure 2. Intracellular retention of the T-cell receptor β chain in CD4+CD8+ double-positive and CD8 single-positive thymocytes of tamoxifen-treated lck-ER^{T2}-SCL transgenic mice. (A) After cell surface staining for CD4, CD8, and TCRβ (anti-TCRβ-FITC, TCRβ surf) thymocytes of tamoxifen (TAM)–treated (7 weeks) lck-ER^{T2}-SCL (n = 3) and wild-type control littermates (n = 3) were permeabilized and subjected to a second staining with anti-TCRβ-PE (TCRβ surf + ic). Anti-TCRβ-PE staining (TCRβ surf + ic) of the permeabilized thymocytes predominantly reflects intracellular staining because surface TCRβ epitopes were already saturated with the TCRβ-FITC antibody (same clone as TCRβ-PE) during the surface staining procedure. Levels of intracellular TCRβ expression (TCRβ surf + ic) were determined within surface DP (double-positive; CD4+CD8+) TCRβ^low, DP TCRβ^high, CD8+TCRβ^low, and CD8+TCRβ^high populations. Representative plots are shown. Dashed lines represent corresponding isotype control data. (B) Quantification (median fluorescent intensity, MFI) of TCRβ surface (surf) and TCRβ intracellular (surf + ic) expression. Bars represent means plus or minus SD; *P < .05; **P < .001. (C) Surface DP TCRβ^high CD8+TCRβ^low cells were analyzed for the presence of intracellular (surf + ic) variable β chains 2, 4, and 14 (Vβ2, Vβ4, and Vβ14). TAM-treated (13 weeks) lck-ER^{T2}-SCL (n = 3) and wild-type control littermates (n = 4) were analyzed. Representative plots are shown.

large CD8+TCRβ^low cells representing CD8 ISP cells were present, however small abnormal CD8+TCRβ^low cells were not detectable. In contrast, 24 hours later abnormal CD8+TCRβ^low cells were unequivocally identified in lck-ER^{T2}-SCL cultures. In parallel, large CD8+TCRβ^low cells representing the CD8 ISP population were evident in both lck-ER^{T2}-SCL and wild-type cultures (Figure 3). Therefore, these data strongly suggest that small CD8+TCRβ^low cells of TAM-SCL thymi were DP derived. Thus, we propose a model in which atypical CD8+TCRβ^low cells result from abnormal differentiation of DP TCRβ^low cells into CD8 SP cells due to the aberrant activation of SCL.

Aberrant SCL activation leads to enhanced survival and decreased proliferation of CD8+TCRβ^low thymocytes

Data regarding apoptosis in constitutive lck-SCL transgenics are conflicting.[12,25] For this reason, we carefully examined apoptosis in TAM-SCL thymi. First, we confirmed in preliminary experiments that TAM treatment did not alter survival of wild-type thymocytes (Figure S4). Next, we performed propidium iodide (PI) analysis of TAM-SCL thymi and TAM-treated wild-type control thymi, which revealed an increased thymic death rate conferred by the activation of SCL (Figure 4A). Subsequently, we investigated early apoptosis in TAM-SCL DP and CD8 SP subpopulations with the mitochondrial dye JC-1.[30] JC-1 multimerizes in charged mitochondrial membranes and emits a red fluorescence. Early apoptosis is associated with loss of the mitochondrial membrane potential (Δψ) leading to decreased JC-1 red fluorescence. As a positive control for apoptosis, we used wild-type thymocytes, which were incubated in vitro at 37°C for 24 hours. We found that an increased percentage of TAM-SCL DP and SP thymocytes was characterized by a decreased Δψ, indicating these cells had initiated apoptosis (Figure 4B). To analyze apoptosis within DP TCRβ^low and CD8+TCRβ^low cells, we performed costaining with annexin V and PI (Figure 4C,D). In contrast to TAM-SCL DP TCRβ^low cells that displayed increased apoptosis, we observed a decreased apoptotic rate within TAM-SCL CD8+TCRβ^low cells (Figure 4C,D). This might explain why CD8+TCRβ^low cells prevail in TAM-SCL thymi.

Next, we intended to examine whether the small size of TAM-SCL DP TCRβ^low and CD8+TCRβ^low cells reflected their low proliferative rate. Normal DP TCRβ^low cells consist of a sizable proportion of large cycling cells.[31] We studied proliferation using the thymidine analog BrdU. Others have shown that after a 24- to 48-hour BrdU exposure, approximately 50% of DP thymocytes had incorporated BrdU in vivo.[32] Analogously, we sought to reach an approximately 50% BrdU incorporation rate by exposing TAM-SCL mice and control mice to BrdU for 36 hours. As expected, we found a dramatic decrease of cycling TCRβ^low DP thymocytes within TAM-SCL mice. This decrease was restricted to TCRβ^low DP thymocytes and was not evident in DP TCRβ^intm and DP TCRβ^high cells (Figure 4E,F). In concordance with their small size, TAM-SCL CD8+TCRβ^low cells were almost not cycling at all. In the 36-hour BrdU exposure period, only 3% of the TAM-SCL CD8+TCRβ^low cells entered the cell cycle. In contrast, around 70% of wild-type CD8+TCRβ^low cells had proliferated in the 36-hour BrdU exposure period (Figure 4E,F). In conclusion, these data demonstrate that SCL increases apoptosis in all populations except CD8+TCRβ^low cells and decreases proliferation of immature thymocytes.
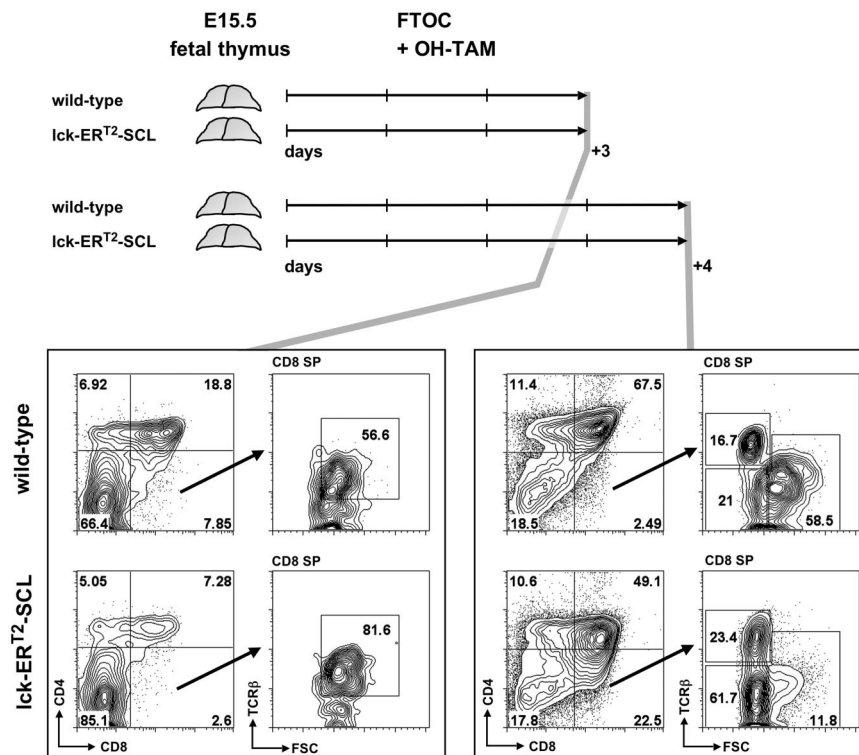
Figure 3. Fetal thymic organ culture revealed the derivation of small CD8+TCRβlow thymocytes from the double-positive CD4+CD8+ stage of T-cell development. Embryonic day–15.5 thymi of lck-ER[T2]-SCL and wild-type littermate control embryos were cultured for 3 and 4 days in the presence of 100 nM hydroxy-tamoxifen (OH-TAM). Representative plots of 3 independent experiments per group are shown. FTOC indicates fetal thymic organ culture; SP, single-positive; and FSC, forward scatter.

Conditional SCL activation leads to increased NOTCH1 pathway activation in thymocytes

Recently, it was shown that a high proportion of SCL-associated human T-ALL lymphoblasts harbored activating *NOTCH1* mutations.[18] Furthermore, it was shown that more than 70% of leukemias arising in SCL transgenic mice also possessed *NOTCH1* mutations.[19,20] It remains unclear at which developmental stage of T-lymphopoiesis *NOTCH1* mutations are acquired. We hypothesized that these mutations might be acquired and may function within the abnormal DP and CD8+TCRβlow populations of SCL-perturbed T-cell development described here. A requirement for *NOTCH1* mutations to function within these populations would be that NOTCH1 is in fact expressed. Previous studies have demonstrated low level NOTCH1 in normal DP thymocytes.[33,34] Therefore, we sought to determine whether TAM-SCL DP TCRβlow and CD8+TCRβlow thymocytes expressed NOTCH1 by flow cytometry. Unexpectedly, we found that NOTCH1 expression in wild-type as well as TAM-SCL DPs was restricted to the immature TCRβlow population (Figure 5A). Remarkably, we observed a significant increase of NOTCH1-expressing cells within TAM-SCL DP TCRβlow cells compared with wild-type (Figure 5A,B). Moreover, a population expressing intermediate levels of NOTCH1 (NOTCH1intermediate) was present in TAM-SCL DP TCRβlow cells, which was nearly absent in wild-type controls (Figure 5A,B). Next, we investigated whether abnormal CD8+TCRβlow cells expressed NOTCH1. Indeed, we were able to identify a small subpopulation of cells (1.7% ± 0.2% [mean ± SD], n = 4) expressing NOTCH1 (Figure 5C). Subsequently, we investigated whether NOTCH1 expression of TAM-SCL preleukemic DP TCRβlow cells mirrored NOTCH1 expression levels within TAM-SCL malignant T-cell lymphoblasts. This analysis was carried out with primary TAM-SCL T lymphoblasts and derived cell lines (Table 2). All primary samples and cell lines expressed high levels of NOTCH1 (Figure 5D; Table 2) similar to the NOTCH1intermediate subpopulation of TAM-SCL DP TCRβlow cells. In addition, we also found NOTCH1

mRNA to be overexpressed in cell lines compared with wild-type thymocytes (Table 2). Next, we investigated whether NOTCH1 up-regulation was associated with *NOTCH1* mutations and sequenced NOTCH1 HD and PEST domains in the derived cell lines. Only 1 of 3 available cell lines displayed a *NOTCH1* mutation (Table 2). Therefore, we concluded that NOTCH1 up-regulation is not necessarily linked to the presence of a *NOTCH1* mutation.

To explore whether the observed preleukemic up-regulation of NOTCH1 results in increased NOTCH1 pathway activation, we studied the expression of the NOTCH1 target genes Hes1 and Deltex1 in preleukemic TAM-SCL thymocytes compared with wild-type controls. We found significantly increased mRNA levels of NOTCH1 and its transcriptional targets Hes1 and Deltex1 in TAM-SCL thymocytes (Figure 6A). Remarkably, the up-regulation of these NOTCH1 targets was already observed after a short-term SCL activation period of 11 days (Figure 6A). Consistent with this, expression of CD25, also a known NOTCH1 target,[35,36] was dramatically increased in TAM-SCL thymocytes (Figure 6B). In concordance with early Hes1 and Deltex1 activation, increased CD25 expression was already present 11 days after the initiation of SCL activation (data not shown). These data imply that aberrantly activated SCL can result in NOTCH pathway activation in arising T lymphoblasts irrespective of the presence of secondary genetic events.

Discussion

The exact mechanism by which deregulated SCL activation in the thymus causes the development of T-ALL remains unclear. In this study, we generated a conditional model of SCL-induced T leukemogenesis to carry out an analysis of T lymphopoiesis in the preleukemic phase. In particular, we were aiming to define possible abnormal cellular compartments induced by SCL, which potentially might be targets of secondary genetic hits required for the
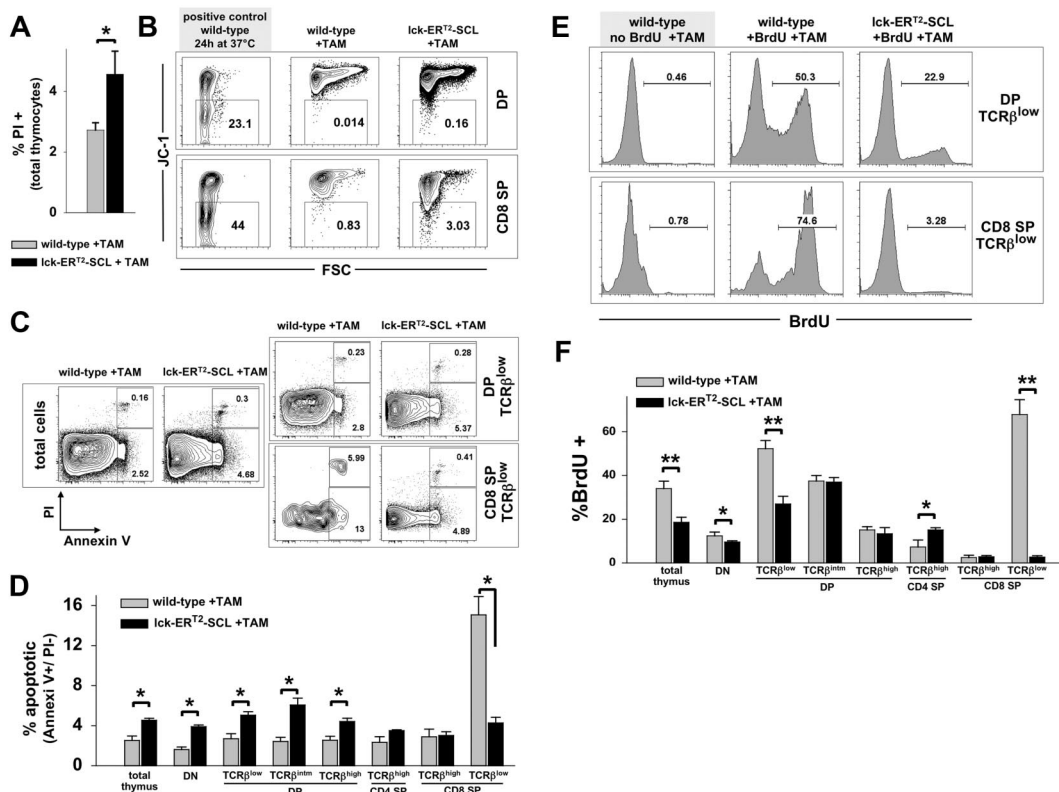
Figure 4. Conditional SCL activation results in decreased apoptosis and decreased cycling of CD8$^+$TCRβ^{low} thymocytes. (A) The proportion of dead thymocytes was examined by propidium iodide (PI) staining. Wild-type, n = 4; lck-ER^{T2}-SCL, n = 4; TAM feed for 12 weeks. Error bars represent means plus or minus SD. (B) Early apoptosis was investigated by the loss of the mitochondrial membrane potential (Δψ), depicted by the loss of JC-1 red fluorescence. The proportion of JC-1–negative (early apoptotic) cells was established within forward and side scatter gated DP (CD4$^+$CD8$^+$, double-positive) and CD8 single-positive (SP) thymocytes. Wild-type, n = 3; lck-ER^{T2}-SCL, n = 3; TAM feed for 11 weeks. (C) The proportion of apoptotic thymocytes (annexin V$^+$/ PI$^-$) was determined within forward and side scatter gated cells. Representative plots of indicated populations are shown. Wild-type, n = 3; lck-ER^{T2}-SCL, n = 3; TAM feed for 7 weeks. (D) Quantification of annexin V/ PI flow cytometric analysis. Bar graphs represent the proportions (± SD) of apoptotic (annexin V$^+$/ PI$^-$) cells within thymic subsets. Error bars represent means plus or minus SD. (E) The proliferation of thymocytes was measured by in vivo BrdU incorporation. Mice (treated with TAM for 5 weeks) were exposed to BrdU for 36 hours before thymus harvest. Representative plots are shown. (F) Quantification of BrdU flow cytometric analysis. Wild-type, n = 4; lck-ER^{T2}-SCL, n = 3; TAM feed for 5 weeks. Bars represent means plus or minus SD; *$P <$ .05; **$P <$ .001. SP indicates single-positive cells.

development of overt malignancy. The use of conditional transgenic models allows the analysis of the initiation, progression, and maintenance of malignant diseases.[37] Herein, we developed a conditional transgenic model (lck-ER^{T2}-SCL) by creating a fusion protein between SCL and the mutated ligand-binding domain of the human estrogen receptor (ER^{T2}).

Using TAM-SCL transgenic mice, we discovered a previously unrecognized thymic cellular pathway that was initiated and sustained by SCL in the preleukemic phase. Nuclear presence of SCL resulted in the increased intracellular accumulation of the TCRβ chain in immature DP thymocytes reflecting the failure of normal TCRαβ complex expression on the cell surface. This failure might render TAM-SCL DPs unable to progress normally through the upcoming selection and lineage decision process and presumably resulted in the increased apoptosis of these cells. However, SCL instructed surviving cells to develop into abnormal noncycling CD8$^+$TCRβ^{low} cells. We speculate that enhanced NOTCH signaling mediated by SCL might be responsible for driving surviving immature DP cells toward the CD8 SP phenotype.[36,38] SCL significantly increased the proportion of NOTCH1-expressing cells within immature DP thymocytes and further induced increased expression of NOTCH1 target genes. These data are indicative of increased NOTCH pathway activation mediated by SCL. In summary these data argue for a cellular pathway in which abnormal immature DP and CD8$^+$TCRβ^{low} cells with activated NOTCH signaling are constantly generated from DN precursors (Figure 6E).

The inability of adequate TCRαβ surface expression in TAM-SCL might be due to the suppression of normal TCRα chain generation by SCL. It has been shown that SCL in combination with LMO1 is capable of inhibiting TCRα rearrangement and expression.[6,39] Moreover, it was demonstrated that SCL interferes with the normal function of the TCRα enhancer.[40] Therefore, it is likely that the inability of SCL transgenic thymocytes to express an assembled surface TCRαβ complex was due to the lack of functional TCRα chain expression.

We used the sequential analysis of TAM-SCL FTOCs to reveal that DP thymocytes emerged before abnormal CD8$^+$TCRβ^{low} cells. If TAM-SCL CD8$^+$TCRβ^{low} cells represented perturbed CD8 ISP cells, they should have appeared before DP thymocytes in the FTOCs. Therefore, we conclude that abnormal TAM-SCL CD8$^+$TCRβ^{low} cells must be derived from DP thymocytes rather than CD8 ISP cells. Strikingly, this abnormal cellular pathway perfectly explains an observation made by Asnafi et al in human SCL-expressing T-ALL samples.[41] This group found that the majority of SCL-associated T-ALL cases presented with a DP or CD8 SP phenotype. CD4 SP T-ALL cases were not observed at all. In agreement with our data, the conclusion by those investigators was that SCL induced T leukemogenesis by inhibiting DP to CD4 rather than inhibiting DN to DP transition. In contrast to most inbred mouse strains, human DN to DP transition progresses via a CD4 ISP rather than a CD8 ISP stage.[42] Therefore, the fact that CD4 SP SCL-expressing T-ALLs were not observed argues against
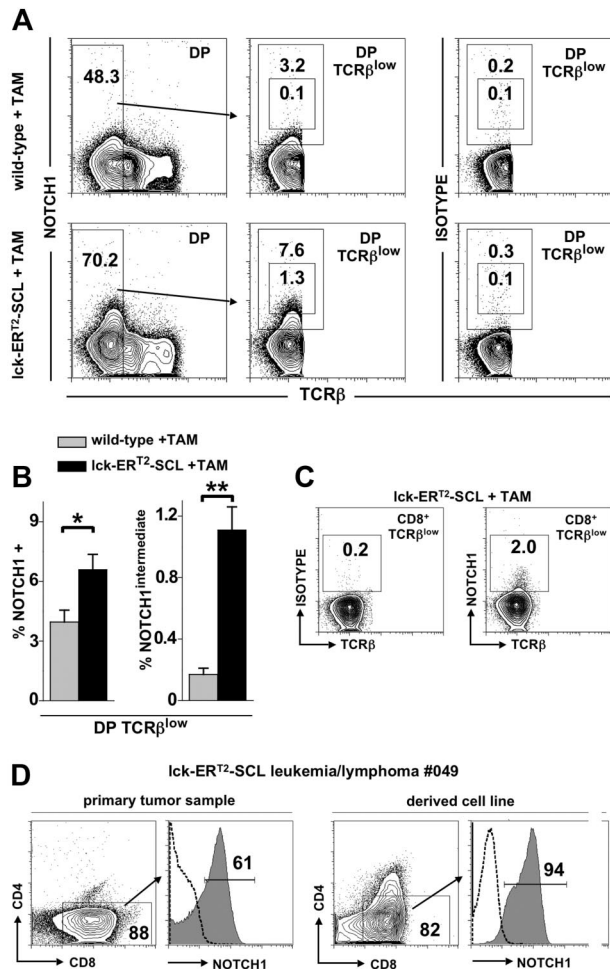
Figure 5. Conditional SCL activation leads to increased NOTCH1 expression. (A) Flow cytometric analysis of CD4^{+}CD8^{+} (double-positive, DP) TCRβ^{low} thymocytes for the expression of NOTCH1 with an antibody (clone mN1A) specific for the intracellular domain of NOTCH1. In addition, analysis with the corresponding IgG[1]κ isotype control antibody is also shown (right). Mice received tamoxifen (TAM) feed for 6 weeks. (B) Quantification of NOTCH1 expression within DP TCRβ^{low} cells of TAM-treated wild-type (n = 4) and lck-ER[T2]-SCL (n = 4) mice. The proportion of total NOTCH1- and NOTCH1[intermediate]-expressing cells was determined and quantified by the gating strategy shown in panel A. *P < .01; **P < .001. (C) Analysis of NOTCH1 expression within the abnormal CD8^{+}TCRβ^{low} population of TAM-treated lck-ER[T2]-SCL mice. (D) Flow cytometric analysis of a primary lck-ER[T2]-SCL tumor (after 11 months of TAM treatment) and the corresponding cell line. Cells were stained for CD4, CD8, and NOTCH1. Dashed lines represent isotype control staining. (A,C,D) Numbers on the plots represent precentages of cells.

Figure 6. Short-term SCL activation induces enhanced NOTCH1 pathway activation. (A) Quantitative real-time PCR analysis of NOTCH1, Hes1, and Deltex1 transcripts from lck-ER[T2]-SCL (n = 3) mice compared with wild-type control littermates (n = 4); TAM treatment as indicated. Levels of statistical significance (fold change; *P < .05 and **P < .001) are indicated. (B) Analysis of CD25 expression on total thymocytes, DP TCRβ^{low}, and CD8^{+}TCRβ^{low} cells. Wild-type (n = 3) and lck-ER[T2]-SCL (n = 3) mice were TAM treated for 11 weeks. Representative plots for each group are shown. (C) Proposed model of the effects of conditional SCL activation on T-cell development.

the notion that ISP thymocytes are the cellular target of SCL-mediated transformation. Our data show that SCL functions after a completed DN to DP transition and therefore after TCRαβ lineage commitment. Thus, our model also explains why SCL-associated T-ALLs are predominantly restricted to the αβ lineage.

Thymi of TAM-SCL mice commencing SCL activation at 3 weeks of age mirrored the thymic changes previously documented with transgenic mice that constitutively express SCL. There were decreased total cell numbers, decreased numbers of DP and CD4 SP cells and increased numbers of CD8^{+}TCRβ^{low} cells, previously interpreted as CD8 ISPs cells.[8,43] Thus, the overt preleukemic phenotype of acquired SCL activation in TAM-SCL mice during established T-cell development was not different from the phenotype observed in transgenic mice that constitutively express SCL. However, the data regarding proliferative potential was not the same. In concordance with the observation that SCL induced decreased thymic cellularity and
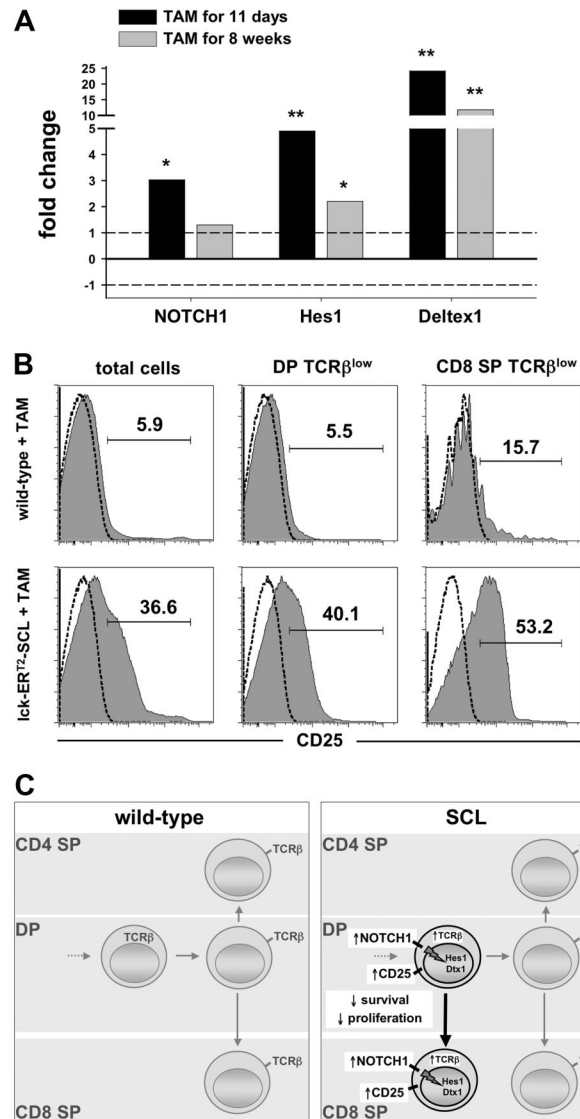
the emergence of small nonlymphoblastic thymocytes, we observed a decreased proliferative activity of total thymocytes. A similar decrease in proliferation was observed within the predominating DN population of preleukemic SCL/LMO1 double-transgenic thymi.[6] In contrast, Shank-Calvo et al observed an 1.7-fold increase of cycling cells in constitutive SCL transgenic thymi.[25] A potential explanation would be that the constitutive SCL transgenic thymocytes had already acquired proliferation-promoting genetic events at the time point of analysis. Interestingly, a proportion of TAM-SCL leukemias demonstrated a CD8^{+}TCRβ^{low} surface phenotype (Table 1), supporting the notion that preleukemic CD8^{+}TCRβ^{low} cells are a leukemic precursor population, and with the acquisition of additional mutations may give rise to a malignant clone.

It is known that NOTCH1 is expressed in normal DP thymocytes,[33,34] however NOTCH1 expression analysis within DP subsets has so far not been performed. Here, we found that NOTCH1 expression within DPs is confined to the immature TCRβ[low] subset. We were able to detect that irregular SCL activation was associated with the up-regulation of NOTCH1 within DP TCRβ[low] thymocytes. This provides a potential link between SCL and NOTCH1 during T-cell leukemogenesis. It has been described that NOTCH1 gain-of-function during T-cell development promotes CD8 and inhibits CD4 SP development.[36] Thus, enhanced NOTCH1 function within SCL-transgenic DP TCRβ[low] thymocytes might be responsible for the development of DPs into CD8 TCRβ cells. Our concept of an enhanced NOTCH1 signal within SCL transgenic DPs provided by the up-regulation of NOTCH1 expression is further supported by increased NOTCH1 target gene activation in TAM-SCL mice. Our observation that the transcriptional NOTCH targets were already up-regulated after a short-term activation period of SCL suggests that the activation of NOTCH signaling is a direct effect of SCL. Whether increased CD25 expression mediates increased IL-2 receptor signaling and might thus be responsible for preventing instant death of immature DP and CD8^{+}TCRβ^{low} cells in TAM-SCL mice remains to be determined. The effect of IL-2 receptor signaling on survival is known to be cellular-context dependent.[44,45] Interestingly however, it has been shown that enhanced IL-2 receptor signaling induced by NOTCH3 in thymocytes is capable of inducing aberrant SCL expression itself.[46] Therefore, NOTCH and SCL might act by mutual amplification during T-cell leukemogenesis.

The link between SCL and NOTCH1 in T leukemogenesis is further underscored by phenotypic similarities between retroviral NOTCH1 gain-of-function models and TAM-SCL mice. Retroviral NOTCH1 gain-of-function leads to a developmental block at the DP stage, which suggested that NOTCH must be down-regulated in DP thymocytes to allow developmental progression to CD4 and CD8 SP cells.[22] Thus, SCL-triggered NOTCH1 up-regulation in DPs might also contribute to the partial developmental block observed at the immature DP stage in TAM-SCL mice. The preleukemic phase of retroviral NOTCH1 gain-of-function models is characterized by accumulating small, noncycling DP thymocytes[47] similar to the noncycling DP cells we observed in TAM-SCL mice. Moreover, increased thymocyte apoptosis was observed within DP and CD8 SP subsets of a constitutive NOTCH1 gain-of-function model very similar to our observations in TAM-SCL mice.[35]

Finally, the lck-ER[T2]-SCL mouse model will provide a valuable tool to study SCL-associated T-cell leukemogenesis. This model will allow novel experimental approaches to address whether the requirement of SCL is confined to the initiation of T-ALL or whether SCL is also required for the maintenance of the malignant phenotype of T-ALL lymphoblasts.

Authorship

Contribution: J.R.G, R.L.B., C.G.B., and D.J.I. designed research; J.R.G, R.L.B., M.S., and D.J.I. performed research; J.R.G., R.L.B., M.S., U.D., C.G.B., and D.J.I. analyzed and interpreted data; J.R.G. and D.J.I. drafted the paper.

Conflict-of-interest disclosure: R.L.B. and C.G.B. are currently employees and shareholders of Amgen. All other authors declare no competing financial interests.

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