Modeling T-cell acute lymphoblastic leukemia induced by the *SCL* and *LMO1* oncogenes

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Deciphering molecular events required for full transformation of normal cells into cancer cells remains a challenge. In T-cell acute lymphoblastic leukemia (T-ALL), the genes encoding the *TAL1/SCL* and *LMO1/2* transcription factors are recurring targets of chromosomal translocations, whereas *NOTCH1* is activated in >50% of samples. Here we show that the *SCL* and *LMO1* oncogenes collaborate to expand primitive thymocyte progenitors and inhibit later stages of differentiation. Together with pre-T-cell antigen receptor (pre-TCR) signaling, these oncogenes provide a favorable context for the acquisition of activating *Notch1* mutations and the emergence of self-renewing leukemia-initiating cells in T-ALL. All tumor cells harness identical and specific *Notch1* mutations and *Tcrβ* clonal signature, indicative of clonal dominance and concurring with the observation that *Notch1* gain of function confers a selective advantage to *SCL-LMO1* transgenic thymocytes. Accordingly, a hyperactive *Notch1* allele accelerates leukemia onset induced by *SCL-LMO1* and bypasses the requirement for pre-TCR signaling. Finally, the time to leukemia induced by the three transgenes corresponds to the time required for clonal expansion from a single leukemic stem cell, suggesting that *SCL, LMO1,* and *Notch1* gain of function, together with an active pre-TCR, might represent the minimum set of complementing events for the transformation of susceptible thymocytes.

[*Keywords*: T-ALL; Notch1; SCL/TAL1; LMO1; leukemia-initiating cell; pre-TCR]

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Studies of human and murine cancers have identified multigenic hits that cooperate in cell transformation (Berns 1988; Hahn and Weinberg 2002). However, deciphering the multistep process that transforms normal cells into fully metastazing cancer cells remains a challenge. Strategies aimed at dissecting cancer genes indicate that they converge to enhance self-renewal, favor growth factor independence, and cause differentiation arrest (Jonkers and Berns 1996; Hanahan and Weinberg 2000). Interestingly, 70% of recurring chromosomal translocations in childhood T-cell acute lymphoblastic leukemia (T-ALL) involve genes encoding oncogenic transcription factors (Look 1997), which are "master" gene regulatory proteins (Rabbitts 1991) in hematopoiesis. These observations suggest that the process of cell transformation subverts cell fate determinants. Recent genome-wide approaches based on gene expression profiling, analysis of copy number alterations, array CGH, or second-generation deep-sequencing technology have identified a number of deletions, new mutations, or dysregulated gene expression in T-ALL (for review, see Mullighan and Downing 2009). These powerful approaches nonetheless likely overlook active, but unmutated, signaling pathways that are essential for the viability of transformed cells (Luo et al. 2009).

Elevated expression of the SCL/TAL1 transcription factor and/or its nuclear partner, LMO1 or LMO2, is found in 50%–60% of childhood T-ALL, associated with gene rearrangements (Begley et al. 1989; Boehm et al. 1991) or not (Ferrando et al. 2002; for review, see Aifantis et al. 2008).

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SCL is a member of the basic helix–loop–helix (bHLH) family of transcription factors that specifies the hematopoietic fate from mesodermal precursors, whereas LMO proteins are zinc-binding LIM domain proteins (for review, see Lecuyer and Hoang 2004). Inappropriate SCL expression in the thymus of *SIL-SCL*, *CD2-SCL*, or *Sca1-SCL* transgenic mice (SCL^{tg}) impairs T-cell differentiation (Larson et al. 1996; Herblot et al. 2000) during the preleukemic stage without inducing leukemia (Aplan et al. 1997; Curtis et al. 1997; Goardon et al. 2002), whereas *Lck-SCL* transgenic mice develop T-cell lymphomas with low penetrance (Condorelli et al. 1996; Kelliher et al. 1996). $LMO1^{tg}$ or $LMO2^{tg}$ mice occasionally develop T-ALL after a long latency period of ~18 mo (Larson et al. 1994). SCL collaborates with LMO1 (Aplan et al. 1997) or LMO2 (Larson et al. 1996) to accelerate disease onset (3–6 mo) and confer full penetrance. While SCL–LMO1/2 are sufficient to initiate leukemogenesis in transgenic mice, other events are required for emergence of full-blown leukemia, as evidenced by a highly variable latency period.

Gain-of-function mutations of the *NOTCH1* gene are found in >50% of human T-ALL (Pear and Aster 2004; Weng et al. 2004) and in $SCL^{tg}LMO1^{tg}$ mouse models of T-ALL (Lin et al. 2006; O'Neil et al. 2006; Gothert et al. 2007). Members of the NOTCH family are evolutionary conserved proteins that control cell fate. NOTCH1 signaling regulates the T-cell versus B-cell fate choice in prethymic progenitors (Sambandam et al. 2005; Aifantis et al. 2008), and also acts at later stages of T-cell development (Zweidler-McKay and Pear 2004). NOTCH1 cooperates with E2A (Ikawa et al. 2006) and the pre-T-cell antigen receptor (pre-TCR) (Ciofani et al. 2004; Aifantis et al. 2008) to specify T-lineage commitment. In the thymus, the pre-TCR (for review, see von Boehmer et al. 1999; Aifantis et al. 2008) controls the critical transition from the $CD4^-CD8^-$ double-negative (DN) to the double-positive (DP; $CD4^+CD8^+$) stages, and coordinates cell proliferation, cell survival, and differentiation in the α/β lineage. In leukemogenesis, the contribution of the pre-TCR appears variable, as it is dispensable in some mouse models (Liao et al. 1998; Engel and Murre 2002) but important in others (Bellavia et al. 2002; dos Santos et al. 2007). In the latter, the precise contribution of the pre-TCR to the leukemogenic process remains unknown. Finally, the importance of the pre-TCR was reported as minor in *SCL–LMO1*-dependent leukemias (Fasseu et al. 2007), although *SCL* expression in childhood T-ALL is invariably associated with high TCR expression (Ferrando et al. 2002). Furthermore, SCL-dependent leukemias in mice harbor in-frame *TCRβ* rearrangements (Chervinsky et al. 2001). Hence, the pre-TCR or TCR pathway may be relevant to the pathophysiology of *SCL*-associated T-ALL.

Cell transformation results from the accumulation of multiple genetic anomalies (Hahn and Weinberg 2002), arguing for the likelihood that the initiating event occurs in a cell with extended proliferative potential, allowing for the acquisition of additional mutations. However, the cell of origin of leukemia need not be a normal stem cell (for review, see Jordan 2009). It was originally thought that ALLs could originate from a committed lymphoid progenitor arrested at the same stage as the bulk of tumor cells. However, oncogenes can modify cellular phenotypes, as observed in several transgenic mouse models in which the DN-to-DP progression can occur in the absence of pre-TCR signaling due to ectopic expression of *BCL2* and *NOTCH1/3* or inhibition of E-protein function (Linette et al. 1994; Engel and Murre 2002; Michie et al. 2007). Furthermore, evidence for the presence of a subpopulation of leukemia-initiating cells (LICs) with stem cell properties in T-ALL remains to be documented.

In the present study, we investigated the cellular and molecular events in *SCL–LMO1*-induced T-ALL using transgenic mice as a model.

Results

The SCL *and* LMO1 *oncogenes expand early thymic precursor (ETP)/DN1 thymocytes*

Circulating bone marrow-derived progenitors ($Kit^+Sca1^+Lin^-$ [KSL]) settle in the thymus, where they give rise to ETPs, a subset of DN1 cells with high T-lineage potential (Sambandam et al. 2005), endowed with the highest proliferative potential in the thymus. ETPs further differentiate into DN2, DN3, and DN4 cells (Fig. 1A). While transition from the ETP to DN2 and DN3 stages is associated with 10-fold expansion in cell numbers at each step, at the DN4-to-DP transition, thymocytes actively cycle under the influence of the pre-TCR, resulting in an ~200-fold expansion of cell numbers (Fig. 1A; Supplemental Fig. S1A) when cells acquire the CD4 and CD8 markers (DP) as well as TCRα/β expression (Fig. 1A). To assess the earliest cell types that are affected by the oncogenes, we first analyzed progenitors in the bone marrow and thymus of $SCL^{tg}LMO1^{tg}$ mice during the preleukemic stage; i.e., at 3–4 wk after birth. The *SCL* transgene is driven by the *SIL* promoter, which is active in all proliferating cells (Supplemental Fig. S1D), whereas *LMO1* expression is driven by the *Lck* transgenic cassette. Expression levels of the endogenous *Lck* and the *LMO1* transgene were assessed by RT–PCR. Both were absent in bone marrow KSL, but expression was initiated in thymic ETP/DN1 cells and was found at higher levels in all subsequent thymocyte subsets (Figs. 1A; Supplemental Fig. S1B,C), indicating that the *Lck* transgenic cassette is active in all thymic precursors, but inactive in bone marrow progenitors. Accordingly, bone marrow KSL numbers were not affected by the transgenes (Supplemental Fig. S2A). In the thymus, however, the *SCL–LMO1* transgenes induced a 10-fold expansion of the ETP population compared with wild-type mice, whereas the variations induced by each transgene separately were not significant (Fig. 1B). This expansion was followed by an expansion of DN3/DN4 populations (Fig. 1C), whereas the DP population was decreased, consistent with previous reports indicating that SCL and LMO1 inhibit thymocyte differentiation (Larson et al. 1996; Aplan et al. 1997; Herblot et al. 2000). Consistent with decreased DP thymocytes, total cell numbers in the thymus of *SCL–LMO1* transgenic mice were decreased compared with age-matched wild-type mice. Thus, the *SCL–LMO1* transgenes induce

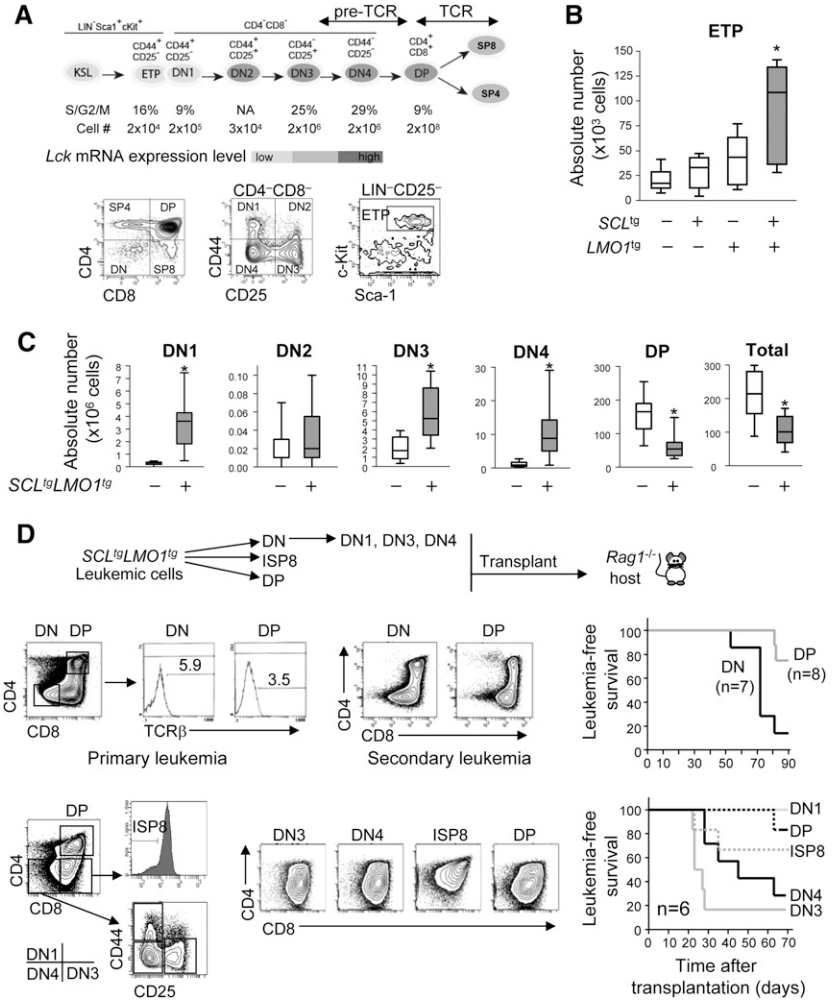


Figure 1. The *SCL* and *LMO1* oncogenes expand the population of ETPs and induce CD3-dependent but antigen-independent T-ALL. (*A*) Schematic diagram of thymocyte populations and their cell surface markers. ETPs are $Kit^+Sca1^+Lin^-CD25^-$. Thymocyte populations (DN1–DN4, DP, SP4, and SP8) are $Thy1.2^+$ and can be further differentiated on the basis of CD25, CD44, CD4, and CD8 expression. The pre-TCR is essential for DN-to-DP transition, whereas the TCR regulates negative and positive selection. Shown are the absolute numbers, the percentages of cells in S/G2/M phase, and *Lck* expression levels as assessed by real-time PCR for each subset of wild-type thymocyte. (*B*) The absolute number of ETPs was assessed by flow cytometry analysis of wild-type, SCL^{tg}, $LMO1^{tg}$, and $SCL^{tg}LMO1^{tg}$ mice at the preleukemic stage (3 wk old). Shown are box plots delimited by the lower and upper 25 percentiles of the distribution with results from Student's *t*-test ([*] $P < 0.001$ compared with wild type). The line inside the box plots represents the median, and those outside represent the two extreme values. (*C*) The absolute number of DN1-to-DP populations was assessed by flow cytometry analysis in wild-type and preleukemic $SCL^{tg}LMO1^{tg}$ mice as in *B*. (*D*) Diagram of the transplantation strategy. Indicated leukemic populations were purified from $Rag1^{+/+}SCL^{tg}LMO1^{tg}$ thymomas and transplanted into immunodeficient $Rag1^{-/-}$ mice at concentrations of 10^3 (DN and DP) or 10^4 (DN1, DN3, DN4, ISP8 and DP) cells. Examples of tumor phenotypes from primary and secondary leukemias, as well as Kaplan-Meier curves of the time to leukemia, are shown.

an expansion of DN thymocytes, starting from the ETP, and inhibit the DN–DP transition.

LICs are enriched in the DN population

At time of overt leukemia, the leukemic population is heterogeneous and comprises DN and DP cells, as reported (Larson et al. 1994; Kelliher et al. 1996; Chervinsky et al. 1999). Leukemia is sustained by self-renewing LICs that can be detected by transplantation into unirradiated immunodeficient mice (Hope et al. 2004). Leukemias arising in $SCL^{tg}LMO1^{tg}$ mice were analyzed for their capacities to initiate secondary leukemias in $Rag1^{-/-}$ mice. We reasoned that the same clonal marker should appear in primary and secondary leukemias, as an indication of self-renewal. Since the rearrangement of the *Tcrβ* locus is a marker of clonality in T cells and of *SCL–LMO1* leukemias (Chervinsky et al. 1999), these leukemias were analyzed for *Vβ8* and *Vβ5* rearrangement by PCR prior to and after transplantation (Supplemental Fig. S3). We found the same rearrangement in primary and secondary leukemias, indicating that the same clone has undergone self-renewal. We next assessed the presence of LIC in leukemic DN and DP populations purified from primary leukemias. Transplantation of 10^6 leukemic cells induced T-ALL in all secondary hosts (data not shown). There was, however, a significant difference among the two groups of mice receiving 10^3 leukemic cells. By 12 wk after transplantation, almost all mice transplanted with DN leukemic cells were moribund, whereas almost all mice grafted with DP leukemic cells remained healthy during this time frame (Fig. 1D). Furthermore, both leukemic DN and DP populations induced secondary leukemias that reproduced the phenotype of the primary tumor in transplanted hosts (Fig. 1D). Therefore, despite an apparent DN–DP hierarchy based on cell surface markers, LICs are found in both DN and DP populations. The leukemia-initiating potential of the DN population was nonetheless higher than that of the DP population, and was found mostly in the DN3 and DN4 populations (Fig. 1D). Even though the preleukemic DN1 population was expanded, the leukemic DN1 population lacks leukemia-initiating potential.

pre-TCR deficiency delays leukemia onset in $SCL^{tg}LMO1^{tg}$ *mice*

The DN3-to-DP transition, which is affected by the transgenes during the preleukemic phase, is controlled

by the pre-TCR. To address the importance of the pre-TCR in cell transformation, we exploited two genetic models in which thymocyte differentiation is blocked at the DN3 stage; i.e., $Cd3\varepsilon^{-/-}$ and $Rag1^{-/-}$ mice.

The pre-TCR and TCR require association with the CD3 complex for signaling (Malissen et al. 1995); therefore, *Cd3*-deficient cells have nonfunctional pre-TCR/TCRs. $SCL^{tg}LMO1^{tg}$ mice develop T-ALL with 100% penetrance (Fig. 2A), as reported (Aplan et al. 1997). However, on a $Cd3\varepsilon^{-/-}$ background, the penetrance of the disease was decreased by 48% (Figs. 2A–4F) and the median survival increased by 234 d when compared with $Cd3\varepsilon^{+/+}$ littermates. Our results indicate a strong genetic interaction between the *SCL–LMO1* oncogenes and *Cd3ε*, but differ to some extent with the report by Fasseu et al. (2007) showing a complete abrogation of *SCL–LMO1*-induced leukemogenesis in $Cd3\varepsilon^{-/-}$ mice. This discrepancy is currently unresolved, but could be due to differences in genetic backgrounds. All of our mice have been backcrossed on a C57BL6 background for >10 generations, excluding the possibility of modifier loci introduced by genetic crossing between $Cd3\varepsilon^{-/-}$ and $SCL^{tg}LMO1^{tg}$ mice. Together, these observations indicate that pre-TCR/TCR signaling collaborates with the *SCL–LMO1* oncogenes to accelerate and increase the penetrance of leukemia.

We took an independent genetic approach with *Rag1*-deficient mice in which T-cell differentiation is arrested at the DN3 stage, because $Rag1^{-/-}$ cells are unable to rearrange their *Tcr* genes and therefore do not express the pre-TCR, the TCRα/β, or TCRγ/δ. *Rag1*-deficient mice expressing the *SCL–LMO1* oncogenes developed leukemia with a similar kinetic as $Cd3\varepsilon^{-/-}SCL^{tg}LMO1^{tg}$ mice (Fig. 2A). Of note, the survival curves for $Rag1^{-/-}SCL^{tg}LMO1^{tg}$ and $Cd3\varepsilon^{-/-}SCL^{tg}LMO1^{tg}$ mice are almost superimposable. Together, our observations concur to indicate the importance of pre-TCR signaling as a collaborating event with the *SCL* and *LMO1* oncogenes.

Major histocompatibility complex (MHC)-mediated antigen presentation and leukemia onset in $SCL^{tg}LMO1^{tg}$ *mice*

Signaling by the pre-TCR and the TCR is important for thymocyte survival and proliferation at the DN and DP stages, respectively. The pre-TCR is antigen-independent (Irving et al. 1998; Gibbons et al. 2001), whereas the activation of TCR signaling is dependent on either MHC class I or MHC class II antigen presentation by antigen-presenting cells (APCs) (Matzinger and Bevan 1977; Marrack and Kappler 1988). Differentiation arrest at the DN3 stage in

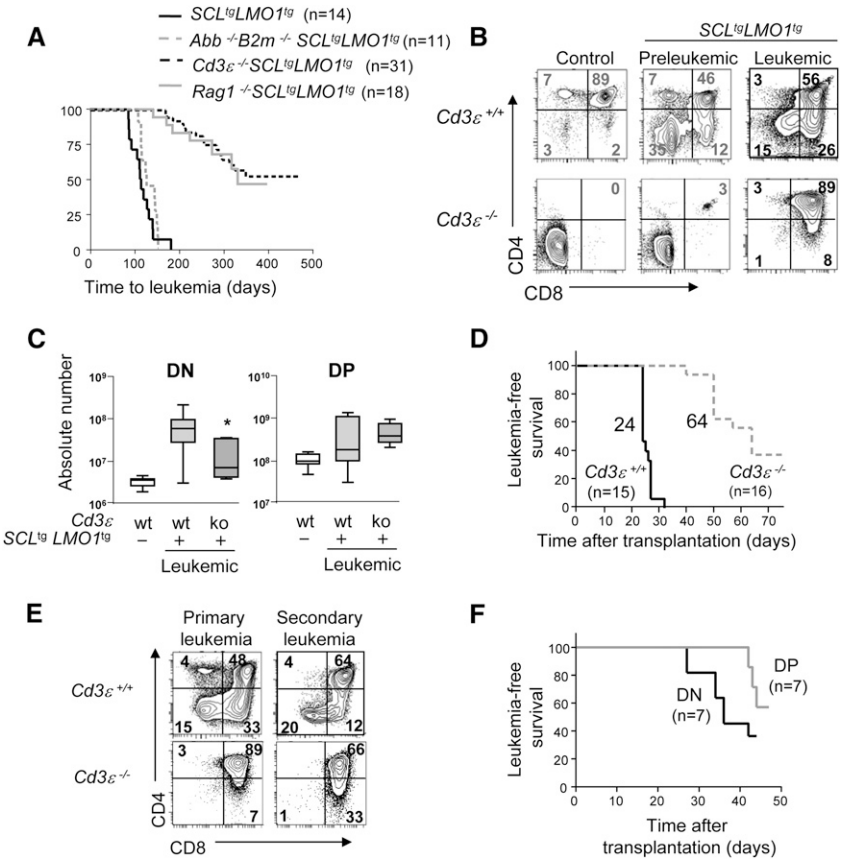


Figure 2. Leukemia initiation is reduced in the absence of CD3/pre-TCR signaling. (*A*) Kaplan-Meier curves of the time to leukemia for $SCL^{tg}LMO1^{tg}$, $Cd3\varepsilon^{-/-}$ $SCL^{tg}LMO1^{tg}$, $Rag1^{-/-}SCL^{tg}LMO1^{tg}$, and $Abb^{-/-}B2m^{-/-}$ $SCL^{tg}LMO1^{tg}$ mice. All malignant thymic lymphomas were diagnosed at necropsy. The median survival times for each cohort of *n* mice are shown. (*B*) Preleukemic and leukemic phenotypes of $SCL^{tg}LMO1^{tg}$ thymocytes. Representative flow cytometry analysis of thymocytes from wild-type and $Cd3\varepsilon^{-/-}$ $SCL^{tg}LMO1^{tg}$ as well as nontransgenic control littermates was performed during the preleukemic stage (4 wk old) and at the time of overt leukemia. Numbers in gray are for preleukemic cells and numbers in black are for overt leukemia phenotype. (*C*) The absolute number of DN and DP populations was assessed by flow cytometry analysis for $Cd3\varepsilon^{+/+}$ $SCL^{tg}LMO1^{tg}$ and $Cd3\varepsilon^{-/-}SCL^{tg}LMO1^{tg}$ tumors. Box plots shown as in Figure 1B ([⋆] $P < 0.05$ as compared with $Cd3\varepsilon^{+/+}SCL^{tg}LMO1^{tg}$ mice). (*D*) Time to leukemia after transplantation of 10^6 leukemic $Cd3\varepsilon^{+/+}$ $SCL^{tg}LMO1^{tg}$ or $Cd3\varepsilon^{-/-}$ $SCL^{tg}LMO1^{tg}$ cells in $Rag1^{-/-}$ mice (*n* mice per group). Shown are the median survival times for each cohort. (*E*) Representative leukemic phenotypes of primary and secondary $Cd3\varepsilon^{+/+}$ and $Cd3\varepsilon^{-/-}$ $SCL^{tg}LMO1^{tg}$ tumors as in *B*. (*F*) Kaplan-Meier curves of the time to leukemia for purified DN or DP leukemic populations from $Cd3\varepsilon^{-/-}SCL^{tg}LMO1^{tg}$ thymomas transplanted into immunodeficient $Rag1^{-/-}$ mice at a concentration of 10^4. Data are shown as in Figure 1D.

Rag1$^{-/-}$ and *Cd3ε*$^{-/-}$ mice did not allow for an assessment of the importance of the TCR in leukemogenesis, which is required later at the DP stage. To directly address the functional importance of the TCR, we examined *SCL–LMO1*-dependent T-ALL in mice deficient for both the *H2-Ab1* gene (*Abb*) and the *β2-microglobulin* gene (*β2m*)—i.e., lacking MHC class I and class II (Grusby et al. 1993)—and therefore deficient for antigen presentation. Unlike *Cd3ε*$^{-/-}$ mice, the kinetics of *SCL–LMO1*-induced leukemia in *Abb*$^{-/-}$*β2m*$^{-/-}$ mice was not affected when compared with wild-type littermates (Fig. 2A), despite impaired differentiation at the DP stage (Supplemental Fig. S4A), presumably due to defective antigen presentation and, hence, defective TCR function. Finally, the phenotype of these leukemia resemble those of *Abb*$^{+/+}$*β2m*$^{+/+}$ *SCL*^{tg}*LMO1*^{tg} mice (Supplemental Fig. S4A). The collaboration between CD3 and the *SCL–LMO1* oncogenes is therefore antigen-independent and, hence, due to pre-TCR signaling. Our observations indicate that antigen-dependent TCR signaling has no discernible contribution to leukemogenesis when the pre-TCR is functional, although we do not exclude the possibility that TCR signaling could be a collaborating event when the pre-TCR is dysfunctional; for example, in the absence of *pTα* (Fasseu et al. 2007).

Cosegregation between leukemia-initiating potential and Cd3 *expression*

To gain further insights into interactions between CD3 and *SCL–LMO1*, we analyzed thymocyte subsets in pre-leukemic and leukemic *Cd3ε*$^{-/-}$*SCL*^{tg}*LMO1*^{tg} mice.

DP cells were absent in *Cd3ε*$^{-/-}$ (Fig. 2B) and *Rag1*$^{-/-}$ (Supplemental Fig. S4A) mice, as expected, due to the lack of pre-TCR signaling. Despite this, transgenic expression of *SCL–LMO1* in *Rag1*$^{-/-}$ and *Cd3ε*$^{-/-}$ mice induced the appearance of a minor population of DP cells (2%–5%) in the thymus during the preleukemic stage, and a dramatic accumulation of DP cells in leukemic mice. Thus, the *SCL* and *LMO1* transgenes allowed for progression to the DP stage independently of the pre-TCR, indicating that the phenotype of leukemic cells in this T-ALL does not reflect the developmental stage of the cell of origin of leukemia. Furthermore, the overwhelming progression to the DP stage at the time of overt leukemia suggests that a subpopulation of DN3 transgenic thymocytes has bypassed the DN–DP checkpoint. Surprisingly, leukemic DN populations were expanded in *Cd3ε*$^{+/+}$ but not in *Cd3ε*$^{-/-}$ tumors, whereas DP populations were more significantly expanded in *Cd3ε*$^{-/-}$ tumors (Fig. 2B,C). In both genotypes, these DP cells are, however, nonfunctional due to the absence of CD3 or of low levels of TCRβ (data not shown).

To assess the presence of LICs in *Cd3ε*$^{+/+}$ and *Cd3ε*$^{-/-}$ leukemias, we transplanted 10^6 leukemic cells in adult *Rag1*$^{-/-}$ mice (Fig. 2D,E). Mice grafted with *Cd3ε*$^{+/+}$ *SCL*^{tg}*LMO1*^{tg} leukemic cells reproducibly developed leukemia within a narrow window of 24–32 d. In sharp contrast, 35% of mice transplanted with *Cd3ε*$^{-/-}$ *SCL*^{tg}*LMO1*^{tg} leukemic cells remained healthy 75 d after transplantation. These observations indicate that *Cd3ε*$^{+/+}$ *SCL*^{tg}*LMO1*^{tg} leukemias have a higher potential to initiate leukemia in secondary hosts.

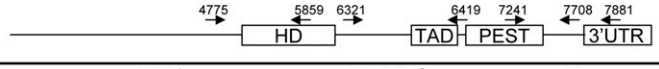
Despite the preponderance of the DP population, we noticed the presence of a minor population of DN cells in *Cd3ε*$^{-/-}$*SCL*^{tg}*LMO1*^{tg} leukemias (Fig. 2B,C). We therefore purified DN and DP cells from these leukemias and found that DN leukemic cells have higher leukemia-initiating potential than DP leukemic cells (Fig. 2F). Furthermore, both DN and DP cells fully reconstitute the phenotypic composition of the primary tumor, giving rise to DP (70%), DN (4%), and immature single positive 8 (ISP8; 15%) cells in transplanted hosts (Supplemental Fig. S4C).

Cosegregation between Cd3 *and* Notch1 *mutations in* SCL*-dependent leukemogenesis*

This high capacity to initiate secondary T-ALL in *Cd3ε*$^{+/+}$ *SCL*^{tg}*LMO1*^{tg} leukemias suggests that additional genetic events might confer stem cell-like properties to populations that are expanded by the *SCL–LMO1* oncogenes. Cytogenetic analysis of tumors from *SCL*^{tg}*LMO1*^{tg} mice by G-banding and SKY (Supplemental Figs. S5, S6) revealed that three out of four tumors exhibit a normal karyotype. One out of four tumors showed the presence of trisomy 7 and/or 16. Thus, the *SCL–LMO1* oncogenes did not cause major chromosomal instability. Nonetheless, the clonal mark of rearranged *Vβ5* and *Vβ8* loci in primary leukemias that was also found in secondary leukemias indicated that the disease is monoclonal, regardless of *Cd3* gene expression (Supplemental Fig. S3). These observations suggest that one or a few clones acquired a proliferative advantage, possibly through the occurrence of additional mutations that were not detected by cytogenetic analysis.

NOTCH1 gain-of-function mutations are frequently found in T-ALL, mostly in the PEST domain and the heterodimerization domain (Weng et al. 2004; Roy et al. 2007; Aifantis et al. 2008). Furthermore, NOTCH signals are required for the generation of ETPs (Sambandam et al. 2005), which we show here to be expanded by the *SCL–LMO1* oncogenes, and to sustain the growth of LICs in human T-ALL (Armstrong et al. 2009). We therefore systematically sequenced the *Notch1* proto-oncogene using cDNAs amplified from six *SCL*^{tg}*LMO1*^{tg} leukemias arising in *Cd3ε*$^{+/+}$ mice and seven in *Cd3ε*$^{-/-}$ mice. We covered sequences upstream of the heterodimerization domain through to the 3′ untranslated region (UTR) in three amplicons (Fig. 3A). Frame-shift insertions and mutations that disrupt the PEST domain of *Notch1* and that are associated with a premature stop were found in *Cd3ε*$^{+/+}$ leukemias, except for sample #241, consistent with published reports (Fig. 3A; Lin et al. 2006; O'Neil et al. 2006). No other mutation was found outside the PEST domain, including the heterodimerization domain. In contrast, no mutation was detected in seven different *Cd3ε*$^{-/-}$ leukemias induced by *SCL–LMO1*. The cosegregation between *Cd3* and *Notch1* gain-of-function mutations suggests a functional link between *Notch1* and *Cd3* in T-ALL.

A

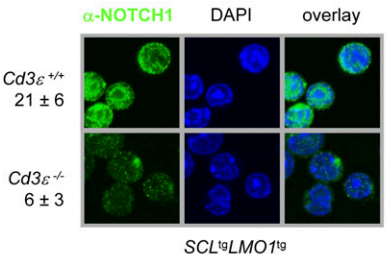


Sample	DNA [1]	Protein [2]	Consequence
Cd3ε +/+ Tg # 3	7051_7052insGTGGT	frameshift starting at AA 2351	premature stop in PEST domain
Cd3ε +/+ Tg # 4	7082delG_insCCTCC	frameshift starting at AA 2361	premature stop in PEST domain
Cd3ε +/+ Tg # 201	7400-7407del_insGGTGAACAA	frameshift starting at AA 2467	premature stop in PEST domain
Cd3ε +/+ Tg # 241	wt	NA	NA
Cd3ε +/+ Tg # 250	7081insG	frameshift starting at AA 2361	premature stop in PEST domain
Cd3ε +/+ Tg # 21929	7192insT	frameshift starting at AA 2398	premature stop in PEST domain
Cd3ε -/-Tg # 1	wt	NA	NA
Cd3ε -/-Tg # 2	wt	NA	NA
Cd3ε -/-Tg # 13	wt	NA	NA
Cd3ε -/-Tg # 1383	wt	NA	NA
Cd3ε -/-Tg # 1951	wt	NA	NA
Cd3ε -/-Tg # 1481	wt	NA	NA
Cd3ε -/-Tg # 1151	wt	NA	NA

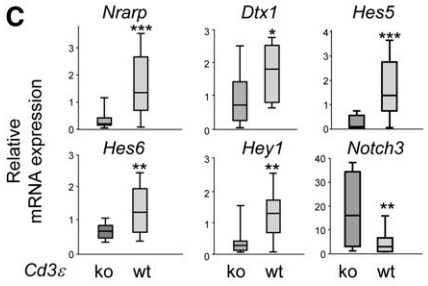
[1] Nucleic acid position according to Ensembl sequence ENSMUSG00000026923

[2] Amino acid position in Notch1 at which mutation occurs

B



C



D

Thymus	Mutation	DN1	DN2	DN3	DN4	ISP8	DP	SP4	SP8
Preleukemic									
#26363	7053insGG (AA2351)	-	-	-	+	+	+	+	+
#26897	7082insC (AA2361)	-	-	-	+	-	+	+	-
#29191	7025insA (AA2342)	-	-	+	+	+	+	+	+
#28806	7099insTCACG (AA2367)	-	-	-	+	+	+	+	+
#28808	7162-7173del_insCACCTCCAGTG (AA2387)	-	-	-	+	+	+	+	+
Leukemic									
#27263	7137insA (AA2379)	-	-	+	+	+	+	+	+
#26888	7214insC (AA2405)	+	+	+	+	+	+	+	+
#29188	7193insGAGG (AA2403)	-	-	-	+	+	+	+	+

( ) Amino acid position in Notch1 at which frameshift occurs

Figure 3. Activating *Notch1* mutations and high NOTCH1 protein levels in *SCL–LMO1* T-ALL are dependent on *Cd3* gene status. (*A*) The *Notch1* gene from $Cd3\varepsilon^{+/+}$ $SCL^{tg}LMO1^{tg}$ and $Cd3\varepsilon^{-/-}$ $SCL^{tg}LMO1^{tg}$ tumors was sequenced in three overlapping amplicons. Shown are the positions of the primers used in the sequencing reactions. HD, TAD, and PEST represent the heterodimerization, transactivation, and protein degradation domains, respectively. (*B*) NOTCH1 intracellular staining in $Cd3\varepsilon^{+/+}$ $SCL^{tg}LMO1^{tg}$ and $Cd3\varepsilon^{-/-}SCL^{tg}LMO1^{tg}$ primary tumor samples. The average fluorescence intensity per pixel from *Z*-stack confocal analysis from two $Cd3\varepsilon^{+/+}SCL^{tg}$ $LMO1^{tg}$ and three $Cd3\varepsilon^{-/-}SCL^{tg}LMO1^{tg}$ different tumor samples is shown as mean ± SD. (*C*) Quantitative RT–PCR of the indicated NOTCH1 target genes were performed in $Cd3\varepsilon^{+/+}SCL^{tg}LMO1^{tg}$ and $Cd3\varepsilon^{-/-}$ $SCL^{tg}LMO1^{tg}$ tumors. Relative mRNA expression levels in tumor cells are normalized by CT values obtained with $Cd3\varepsilon^{+/+}$ $SCL^{tg}LMO1^{tg}$ cells and are presented as box plots with the median and extreme values of each distribution ([*] $P < 0.05$; [**] $P < 0.01$; [***] $P < 0.001$ as compared with $Cd3\varepsilon^{-/-}SCL^{tg}LMO1^{tg}$ tumors). (*D*) The *Notch1* gene from purified late preleukemic and leukemic thymocyte subsets from $Cd3\varepsilon^{+/+}$ $SCL^{tg}LMO1^{tg}$ mice was sequenced as in *A*. (ISP8 corresponds to $Thy1.2^{+}CD8^{+}CD4^{-}Cd3\varepsilon^{lo}$, SP4 corresponds to $Thy1.2^{+}CD4^{+}CD8^{-}$, and SP8 corresponds to $Thy1.2^{+}CD4^{-}CD8^{+}$.)

Cosegregation between Cd3 *expression, NOTCH1 protein levels, and NOTCH1 activity in leukemic thymocytes*

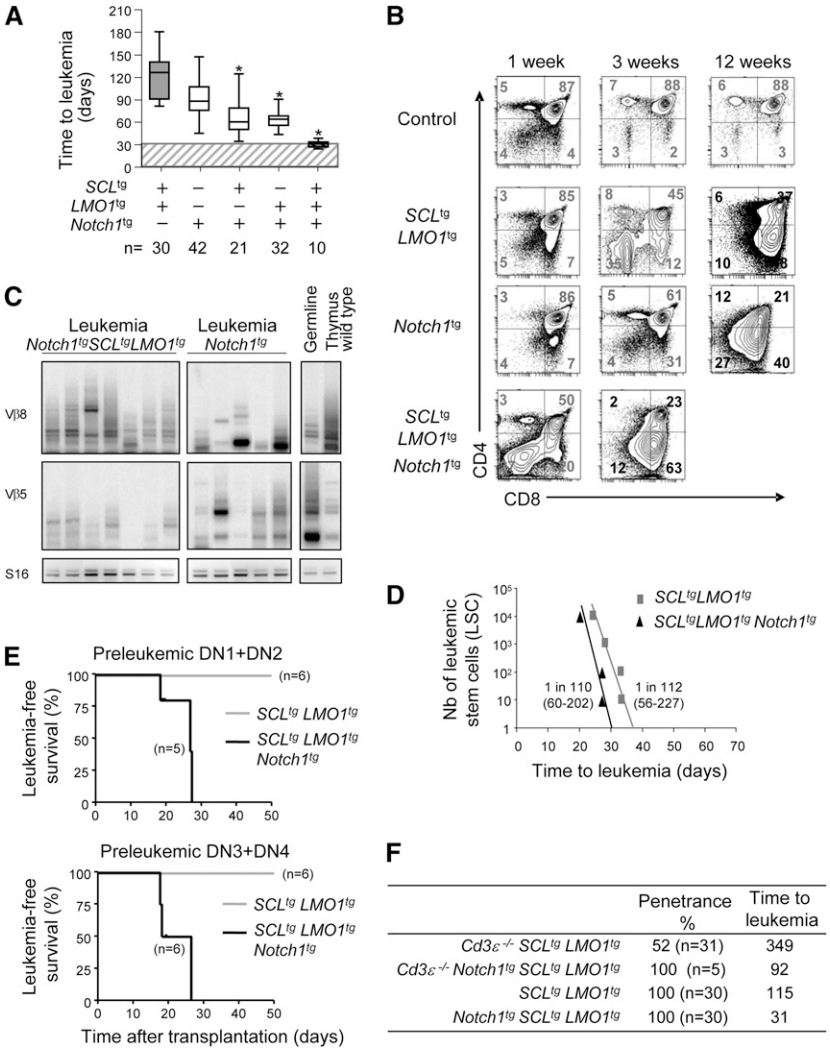
To verify the consequences of mutations in the PEST domain, we first determined NOTCH1 protein levels by immunofluorescence in $Cd3\varepsilon^{+/+}$ and $Cd3\varepsilon^{-/-}$ *SCL–LMO1* leukemias. We found fourfold higher NOTCH1 protein levels in $Cd3\varepsilon^{+/+}$ tumors and strong nuclear staining (Fig. 3B; Supplemental Fig. S7A), indicating that mutations of the PEST domain result in increased NOTCH1 protein. Increased nuclear NOTCH1 in $Cd3\varepsilon^{+/+}$ tumors was accompanied by higher expression of known NOTCH1 target genes in $SCL^{tg}LMO1^{tg}$ leukemias (Fig. 3C; Supplemental Fig. S7B), *Nrarp*, *Dtx1*, *Hes5*, *Hes6*, and *Hey1*. *Hes1*, *Il7r*, *Gata3*, and *Myc* did not differ between $Cd3\varepsilon^{+/+}$ and $Cd3\varepsilon^{-/-}$ leukemias. Finally, *Notch3* and *Ptcra* showed higher expression levels in $Cd3\varepsilon^{-/-}$ leukemias. Since *Hes1*, *Il7ra*, *Myc*, and *Ptcra* are also controlled by NOTCH3 (Bellavia et al. 2002; Ong et al. 2006; Vacca et al. 2006), the elevated levels of *Notch3* in $Cd3\varepsilon^{-/-}$ leukemias may compensate for lower NOTCH1 levels in these leukemias. Of note, *Myc* is expressed in both $Cd3\varepsilon^{+/+}$ and $Cd3\varepsilon^{-/-}$ $SCL^{tg}LMO1^{tg}$ tumors at levels comparable with $Notch1^{tg}$ tumors, consistent with an important role for c-Myc in leukemogenesis downstream from NOTCH1 (Sharma et al. 2006; Weng et al. 2006) and possibly NOTCH3. Furthermore, NOTCH1 target genes were low in leukemic samples from mouse #241, in which the *Notch1* gene was wild type and for which the time of leukemia onset was the longest (183 d) within the *SCL–LMO1* series. These results suggest that NOTCH1 activity is higher in $Cd3\varepsilon^{+/+}SCL^{tg}LMO1^{tg}$ tumors, concurring with higher NOTCH1 protein levels and PEST domain mutations in the *Notch1* gene.

Strikingly, *Notch1* mutations were found in DN4 cells during the late preleukemic stage, but not in more primitive thymocyte progenitors, except for sample #29191 (Fig. 3D). Furthermore, the same mutation was found in subsequent

stages of thymocyte differentiation. However, mutations were found at different positions in five different preleukemic samples. Together, these observations further support the hypothesis that the acquisition of *Notch1* gain of function confers clonal dominance. In sample #26897, we did not observe *Notch1* mutations in the CD8 lineage, possibly because of the limit of detection of the PCR reaction, or, alternatively, these cells are not functional $CD8^+$ cells, but represent more immature stages. These observations indicate that *Notch1* mutations occur mostly at the DN4 stage during the preleukemic phase, and concur with the absence of *Notch1* mutations in *Cd3*-deficient leukemias (Fig. 3A–D). At the time of overt leukemia, however, we found *Notch1* mutations in DN3 cells in two out of three samples. Since DN3 cells are defined as $CD25^+CD44^-$ and, furthermore, CD25 is a target of NOTCH (Deftos et al. 2000), it is possible that the acquisition of *Notch1* gain-of-function mutations may cause abnormal CD25 expression. The presence of *Notch1* mutations in DN1 and DN2 subpopulations in sample #26888 may be due to disrupted hierarchical organization and abnormal differentiation in leukemia (Jordan 2009), or, alternatively, that DN1 cells can occasionally acquire *Notch1* mutations. Again, all subpopulations have the same mutation as observed during the preleukemic phase, consistent with clonal dominance. Together, our observations indicate that *Notch1* gain of function as well as an active pre-TCR collaborate with the *SCL* and *LMO1* oncogenes.

Genetic interactions between Notch1 *and* SCL–LMO1

To determine whether *Notch1* interacts genetically with the *SCL* and *LMO1* oncogenes, we crossed $SCL^{tg}LMO1^{tg}$ mice with mice overexpressing a constitutively active intracellular NOTCH1, lacking the PEST and part of the TAD domains (*Notch1-ICΔP*), under the control of the *Lck* promoter (Fowlkes and Robey 2002). $Notch1^{tg}$ or $SCL^{tg}LMO1^{tg}$ mice developed acute leukemia with highly variable times of onset (Fig. 4A). The *SCL* or *LMO1* transgene did not induce leukemia separately, but significantly



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	Penetrance %	Time to leukemia
Cd3ε$^{-/-}$ SCL^{tg} $LMO1^{tg}$	52 (n=31)	349
Cd3ε$^{-/-}$ *Notch1*^{tg} SCL^{tg} $LMO1^{tg}$	100 (n=5)	92
SCL^{tg} $LMO1^{tg}$	100 (n=30)	115
Notch1^{tg} SCL^{tg} $LMO1^{tg}$	100 (n=30)	31

Figure 4. Genetic interaction between *SCL*, *LMO1*, and a hyperactive *Notch1* allele in leukemogenesis. (*A*) Survival analysis of $SCL^{tg}LMO1^{tg}$, $Notch1^{tg}$, $Notch1^{tg}SCL^{tg}$, $Notch1^{tg}LMO1^{tg}$, and $Notch1^{tg}$ $SCL^{tg}LMO1^{tg}$ mice. Results are presented as in Figure 2A ([⋆] $P < 0.001$ as compared with $SCL^{tg}LMO1^{tg}$ mice). Cohorts of *n* mice were analyzed per genotype. (*B*) Preleukemic and leukemic phenotypes of $Notch1^{tg}$ and $Notch1^{tg}SCL^{tg}$ $LMO1^{tg}$ mice. Representative flow cytometry analysis of thymocytes was performed at the indicated times as in Figure 2B. (*C*) Oligoclonal T-cell expansion detected by PCR analysis of *Tcrβ* gene rearrangements in $Notch1^{tg}$ and $Notch1^{tg}SCL^{tg}LMO1^{tg}$ tumors. Genomic DNA was amplified by PCR with primers for specific variability segments of *Tcrβ*. Shown is germline configuration, rearrangements in wild-type thymus, and seven independent $Notch1^{tg}SCL^{tg}LMO1^{tg}$ and five independent $Notch1^{tg}$ tumors. (*D*) Leukemic cells from $SCL^{tg}LMO1^{tg}$ and $Notch1^{tg}$ $SCL^{tg}LMO1^{tg}$ were transplanted through limiting dilution in $Rag1^{-/-}$ mice. Shown is the time to leukemia after transplantation of the indicated numbers of LSC equivalent. LIC frequency (Range LIC ± SE) for the indicated genotype was calculated by applying Poisson statistics using the Limit Dilution Analysis software (Stem Cell Technologies). (E) Purified preleukemic thymocyte populations from 7-d-old $SCL^{tg}LMO1^{tg}$ and $Notch1^{tg}SCL^{tg}LMO1^{tg}$ mice were transplanted into sublethally irradiated coisogenic Pep3B mice at a concentration of 3×10^4. Shown are the numbers of mice presenting T-ALL 4 wk post-transplantation over the total numbers of transplanted mice. (*F*) Penetrance and median time to leukemia for $SCL^{tg}LMO1^{tg}$, $Notch1^{tg}$ $SCL^{tg}LMO1^{tg}$, $Cd3\varepsilon^{-/-}$ $SCL^{tg}LMO1^{tg}$, and $Cd3\varepsilon^{-/-}Notch1^{tg}$ $SCL^{tg}LMO1^{tg}$.

accelerated leukemia onset in the presence of *Notch1-ICΔP*, suggesting that these genes operate in parallel pathways. In all of these series, the time of onset remains highly variable. Strikingly, triple *Notch1*^{tg}*SCL*^{tg}*LMO1*^{tg} mice developed leukemia almost synchronously around day 31 after birth. This shortened time of onset ($P < 0.01$) indicates synergistic interactions. Furthermore, this narrow window of onset suggests that the introduction of *Notch1-ICΔP* is sufficient to bypass the time required for clonal evolution, which is highly variable.

We next assessed the cellular distribution within the DN population of leukemic cells. In contrast to the *SCL* and *LMO1* transgenes, *Notch1-ICΔP* did not cause an expansion of the DN1/DN2 populations, but of the DN4 and ISP8 (Supplemental Fig. S6C), even though the *Notch1-ICΔP* transgene may be expressed at earlier stages. Since *Notch1* mutations cosegregate genetically with *Cd3* (Fig. 3A), and *Notch1* mutations are found in DN4 cells and later stages, we surmise that the proliferative signal from the pre-TCR increases the likelihood of accumulating mutations that include the *Notch1* locus. In leukemic thymi, coexpression of the *SCL–LMO1* transgenes had expanded the numbers of DN1 and DN2 cells compared with leukemias arising in *Notch1*^{tg} mice, whereas the other populations did not differ significantly between the two types of leukemias. Notably, the heterogenous distribution within each DN or DP subset was markedly reduced in triple-transgenic mice compared with *Notch1*^{tg} or *SCL*^{tg}*LMO1*^{tg} mice (Supplemental Figs. S4B-S6C). In addition, *Cd3ε*$^{-/-}$ leukemias differ most from triple-transgenic mice; i.e., the numbers of DN1 and DN4 cells were lowest and the number of DP was highest, consistent with the absence of *Notch1* mutations in these leukemias. However, the ISP8 population was in the same range, indicating that the *SCL–LMO1* oncogenes can expand this population in the absence of *Notch1* gain-of-function mutation. Overall, the total numbers of cells within the various DN subsets in leukemias arising in triple *Notch1*^{tg}*SCL*^{tg}*LMO1*^{tg} mice was not higher than those arising in *SCL*^{tg}*LMO1*^{tg} or *Notch1*^{tg} mice (Supplemental Fig. S6C). We next verified the clonality of leukemias arising in *Notch1*^{tg}*SCL*^{tg}*LMO1*^{tg} mice and traced the presence of numerous clones (Fig. 4C), contrasting with the predominance of one or a few clones in *SCL*^{tg}*LMO1*^{tg} leukemias (Supplemental Figs. S3, S4B). The presence of multiple clones in leukemias from *Notch1*^{tg}*SCL*^{tg}*LMO1*^{tg} mice suggests that T-ALL occurred in the absence of obvious clonal dominance.

To determine whether the shortened time to leukemia in triple *Notch1*^{tg}*SCL*^{tg}*LMO1*^{tg} compared with *SCL*^{tg}*LMO1*^{tg} mice might be due to increased LIC frequencies, we transplanted *Rag1*$^{-/-}$ mice with limiting dilutions of cells from these leukemias (Fig. 4D). We found that LIC frequency was not increased by the *Notch1* transgene. Furthermore, this limiting dilution analysis indicates that the time to leukemia for one leukemic stem cell (LSC) equivalent is 31 d for *Notch1*^{tg}*SCL*^{tg}*LMO1*^{tg} leukemias and 36 d for *SCL*^{tg}*LMO1*^{tg} leukemias. These similarities concur with the presence of spontaneously occurring *Notch1* mutations in *SCL*^{tg}*LMO1*^{tg} leukemias and with the determining importance of these gain-of-function mutations on the biology of the leukemic clone. Strikingly, this time to leukemia was comparable with that observed in *Notch1*^{tg}*SCL*^{tg}*LMO1*^{tg} mice (Fig. 4A). We therefore conclude that the 92-d difference between the median time to leukemia of double *SCL*^{tg}*LMO1*^{tg} and triple *Notch1*^{tg}*SCL*^{tg}*LMO1*^{tg} mice represents the time required for acquisition of *Notch1* mutations in a *Cd3ε*$^{+/+}$ background.

To address the question of whether a constitutively active *Notch1* gene confers leukemia-inducing potential to preleukemic *SCL–LMO1*-expressing DN or DP cells, we transplanted 3×10^4 purified DN1–DN2 and DN3–DN4 cells and 3×10^5 DP cells from double *SCL*^{tg}*LMO1*^{tg} and triple *Notch1*^{tg}*SCL*^{tg}*LMO1*^{tg} mice into sublethally irradiated hosts (Fig. 4E; data not shown). The results show that purified thymocyte subsets from double *SCL*^{tg}*LMO1*^{tg} mice failed to induce leukemia after 70 d, indicating that these two oncogenes are not sufficient for cell transformation. This contrasts with the capacity of leukemic DN3/4 cells to initiate leukemia in transplanted hosts, possibly due to acquired *Notch1* mutations. Indeed, purified DN1–DN2 and DN3–DN4 cells from triple *Notch1*^{tg}*SCL*^{tg}*LMO1*^{tg} mice induced T-ALL in secondary recipients with high efficiency, indicating that *Notch1* gain of function confers leukemogenicity to *SCL–LMO1*-expressing DN thymocytes. In contrast, the DP population was much less susceptible, and only one of six recipient mice developed leukemia. Moreover, the transformation of DN1–DN2 cells by the three oncogenes indicates that pre-TCR is not essential for leukemogenesis. Accordingly, we observed that the *Notch1* transgene allows for a bypass of pre-TCR signaling deficiency to induce T-ALL with full penetrance and shortened latency on a *Cd3*-deficient background (Fig. 4F). Finally, all preleukemic triple-transgenic DN subsets induced T-ALL within 25 d, which corresponds to the time required for clonal expansion of fully transformed LSCs. Together, these experiments indicate that the three oncogenes—*Notch1*, *SCL*, and *LMO1*—are sufficient to convert susceptible thymocyte precursors into LICs without requiring pre-TCR signaling.

Our observations concur to support the conclusions that the variable times of leukemia onset in *Cd3ε*$^{+/+}$ *SCL*^{tg}*LMO1*^{tg} mice represents the time required for accumulation of *Notch1* mutations (Fig. 5A). Accordingly, the presence of all three oncogenes at birth is sufficient for full transformation of primary thymocyte precursors. The time to leukemia therefore represents the time required for clonal expansion (Fig. 5B).

Discussion

In the present study, we provide genetic evidence that *Notch1* and the pre-TCR collaborate with the *SCL–LMO1* oncogenes in T-ALL.

The pre-TCR and Notch1 as collaborating events in leukemogenesis

Primary oncogenic events such as those triggered by chromosomal translocations are generally insufficient by themselves to cause leukemias, and require secondary cooperative mutations to fully transform cells. These collaborating

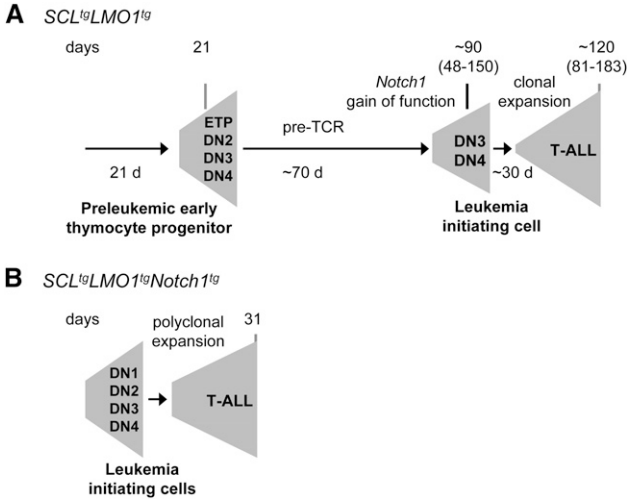

Figure 5. Model of progression to leukemia. (*A*) At the time of weaning, thymic expression of the *SCL–LMO1* oncogenes induces an expansion of the ETP/DN1 to DN4 population. A period of 92 d is necessary for acquisition of *Notch1* gain-of-function mutations. The clonal expansion of the leukemic population takes another 31 d before overt leukemia. (*B*) The presence of all three oncogenes (*Notch1*, *SCL*, and *LMO1*) at birth is sufficient for full transformation of primary thymocyte precursors. The time to leukemia therefore represents the time required for clonal expansion.

events may be different, depending on the initiating cytogenetic event and the target cell of transformation. Hence, T-ALL with *MLL* rearrangements exhibit high *Flt3* levels (Armstrong et al. 2002; Brown et al. 2005), whereas *NOTCH1*-activating mutations were found in most molecular subgroups of T-ALL (Weng et al. 2004; Grabher et al. 2006).

Previous work indicates that pre-TCR or TCR signaling (Chervinsky et al. 2001; Fasseu et al. 2007) accelerates *SCL*-dependent leukemias in transgenic mouse models, and that SCL$^+$ T-ALL constitutes a poor prognostic group in childhood leukemias (Ferrando et al. 2002), associated with elevated expression of genes of the TCR pathway. The relative importance of the pre-TCR or the TCR, however, remains debatable. Here, we show that abrogation of antigen presentation in the $Abb^{-/-}\beta2m^{-/-}$ mice did not delay *SCL–LMO1*-induced leukemias, indicating that, when the pre-TCR is active, the contribution of antigen-dependent TCR signaling to leukemogenesis is minimal. In contrast, CD3 signaling is an important determinant, consistent with the report that all *SCL–LMO1*-induced T-ALL in SCID mice have an in-frame rearranged TCRβ and express the pTα (Chervinsky et al. 2001), suggesting a functional importance for the pre-TCR. Nonetheless, when the pre-TCR is inactive, as in *pTα*-deficient mice in which TCRα/β lymphocytes are still produced, albeit at reduced levels, it is possible that the TCRα/β or TCRγ/δ may provide an alternative collaborative signal for the *SCL–LMO1* oncogenes, as reported (Fasseu et al. 2007).

Despite the recognition that pre-TCR signaling is important in T-ALL, the precise contribution of the pre-TCR to leukemogenesis remains to be documented. In the present study, we provide evidence that the aggressiveness of pre-TCR-proficient *SCL–LMO1* T-ALL is associated with *Notch1* mutations. Activating *Notch1* mutations are a frequent occurrence in T-ALL and have been shown to coincide with the appearance of pre-LSCs in *SCL*^{tg}*LMO1*^{tg} mice (Lin et al. 2006). The absence of *Notch1* gain-of-function mutations in $Cd3\varepsilon^{-/-}$ leukemias suggest that either *Notch1* mutations do not provide a selective advantage to pre-TCR-deficient cells, or these cells are not permissive to *Notch1* mutations. Here we provide several lines of evidence in support of the second hypothesis. First, we show that SCL–LMO1 expands ETP-DN1 cells and subsequent DN stages of thymocyte differentiation but inhibits the DN4–DP transition, thus favoring the accumulation of cells at the highly proliferative DN4 stage and, consequently, the appearance of additional mutations. Second, we found that activating *Notch1* mutations cosegregate with *Cd3ε* in leukemogenesis. Third, in *Cd3ε*-proficient cells, *Notch1* mutations are found in DN4 cells and later maturational stages, but not in DN1/2 and rarely in DN3 cells. Nonetheless, a hyperactive *Notch1* allele bypasses the requirement for pre-TCR function in SCL–LMO1-induced leukemias. In summary, our observations indicate that the pre-TCR provides a permissive context for the appearance of mutations that include gain-of-function mutations of the *Notch1* gene in primary thymocyte precursors expanded by the *SCL–LMO1* oncogenes. Finally, our observation that, during the preleukemic phase, this hyperactive *Notch1* allele confers leukemogenic potential to *SCL*^{tg}*LMO1*^{tg} DN1–DN2 subsets that are pre-TCR-independent indicates that *Notch1* can provide a selective advantage in the absence of the pre-TCR (Campese et al. 2006).

A recent study indicates that LMO2 but not *Notch3* favors the self-renewal of DN3 thymocytes (McCormack et al. 2010), leaving open the question of the role of *Notch1* gain-of-function mutations in T-ALL. Our observations indicate that activating *Notch1* mutations are essential for cell transformation induced by the *SCL* and *LMO1* oncogenes. Previous work places the *Myc* oncogene downstream from NOTCH1 (Sharma et al. 2006; Weng et al. 2006; Li et al. 2008). As a corollary, *Notch1* and *Myc* mutations are mutually exclusive in large-scale insertional mutagenesis screens (Kool and Berns 2009). Furthermore, *SCL*-induced leukemias are also accelerated by loss of *Trp53* (Curtis et al. 1997), and *Notch1* has been shown to suppress *p53* in lymphomagenesis (Beverly et al. 2005), consistent with the observations that activating *Notch1* mutations are not found in *p53*-deficient lymphomas (Uren et al. 2008). It will be interesting to assess whether p53 deficiency or *c-Myc* acts downstream from or in parallel to *Notch1* in *SCL–LMO1/2*-induced leukemias. Although *Notch1* mutations occur at a high frequency of ~80% in $Cd3\varepsilon^{+/+}$*SCL*^{tg}*LMO1*^{tg} mice (Lin et al. 2006; this study), cell transformation can also involve alternative pathways, such as the Wnt/β-catenin signaling pathway (Guo et al. 2007).

Target cell of transformation

The nature of the target cells of transformation by oncoproteins remains an open question. The DP population is

transient in the thymus, and most DP thymocytes are eliminated by apoptosis. Thus, even if oncogene activation can occur in these cells, the probability of survival and subsequent clonal evolution is limited. Our genetic approach, combined with transplantation of purified thymocyte progenitors during the preleukemic phase (i.e., 1 wk after birth), indicates that the collaboration between the three oncogenes (*SCL*, *LMO1*, and *Notch1*) is sufficient to transform susceptible target cells; i.e., DN1–DN4 thymocyte progenitors. In principle, all hematopoietic progenitors that can give rise to thymocytes could be targeted by the *Notch1* oncogene. Indeed, retroviral delivery of a constutively active *NOTCH1* gene in hematopoietic stem cells (HSCs) has been shown to exclusively induce T-ALL (Pear et al. 1996). In contrast, our observations indicate that DP cells are less susceptible to cell transformation by these oncogenes. Since the DP stage represents a critical checkpoint in the thymus, it is possible that only DP cells that have successfully undergone selection are prone to oncogene transformation, whereas cells that are fated to be eliminated may require additional collaborating events.

Since SCL–LMO1/2 leukemias are mostly DP, these observations are consistent with the view that the surface phenotype of the bulk of leukemic cells may not be indicative of the cell of origin of leukemia. This is further supported by our genetic approach that ectopic expression of the *SCL–LMO1* oncogenes in the thymus of *Rag1*- or *Cd3ε*-deficient mice induces tumors that "illegitimately" express the CD4 and/or CD8 markers (DP or ISP8), even though these thymocytes should be arrested at the DN3 stage due to pre-TCR deficiency.

Similar to *Notch1*, ectopic *LMO2* expression in bone marrow HSCs by retroviral gene transfer (our unpublished results) or by LTR-mediated activation of the endogenous gene (for review, see Hoang 2010) also gives rise to T-ALL. Thus, bone marrow-derived HSCs and all DN thymic populations are susceptible to transformation by NOTCH1, SCL, and LMO1. Nonetheless, in the double *SCL–LMO1* transgenic mouse model, *Notch1* mutations accumulate at the DN4 stage, possibly due to intensive cell proliferation. Since *Notch1* gain of function confers leukemia-initiating potential to *SCL–LMO1* transgenic thymocytes, it is quite possible that *SCL–LMO1* favors the accumulation of DN4 cells by expanding the DN1 population, but also by preventing the DN4–DP transition, thereby facilitating the acquisition of complementing *Notch1* mutations.

LSCs have been studied extensively in acute myeloid leukemia (AML), where they are found in primitive populations (Lapidot et al. 1994). In contrast, the identification of LSCs in ALL is still controversial (Jordan 2009). A recent study in human B-ALL showed that most populations of leukemic blasts were able to reconstitute the tumor phenotype (le Viseur et al. 2008). Transformed leukemic cells could follow a disorganized differentiation pattern associated with an illegitimate expression of surface markers resulting from oncogenic protein misexpression, such as *NOTCH1/3* (Michie et al. 2007). Despite the fact that preleukemic DP cells rarely initiate leukemias in transplanted hosts, we show here that both leukemic DN and DP populations in *SCL–LMO1* leukemias have leukemia-initiating potential, even though leukemic DN3–DN4 cells have the highest potential in our model. During the preleukemic phase, *Notch1* mutations occur in DN4 thymocytes. At time of overt leukemia, however, *Notch1* mutations were identified in leukemic cells with a more primitive phenotype, which could be explained by either a developmental flexibility (Jordan 2009) or oncogene-mediated surface marker misexpression. Indeed, in $Notch1^{tg}$ thymocytes, we observed an increase in *Cd25* expression (Deftos et al. 2000; Fowlkes and Robey 2002; data not shown) that could confer a DN3 surface phenotype to cells that are actually at the DN4 stage. Together, our results show that different subpopulations of leukemic cells have leukemia-initiating potential, suggesting that leukemic cells in T-ALL do not follow an orderly developmental structure.

Significance of the time to leukemia

In our transgenic model, *SCL* and *LMO1* gains of functions precede the acquisition of *Notch1* mutations (Fig. 5; Lin et al. 2006), although the sequence of events may also be inverted, as reported in some cases of childhood T-ALL (Eguchi-Ishimae et al. 2008; Hong et al. 2008). It has been proposed that acquiring the appropriate numbers of complementary genetic hits may be more important than the sequence of events in cell transformation, even though these genetic alterations may occur according to a preferred sequence (Fearon and Vogelstein 1990). In the present study, we provide several lines of evidence supporting the hypothesis that *Notch1* gain-of-function mutations confer clonal dominance to *SCL–LMO1* transformed cells. First, the clonality of leukemic cells as assessed by the *Tcrβ* rearrangement indicates the emergence of a single clone. Second, seven of eight *Cd3ε*-proficient leukemias exhibit *Notch1*-activating mutations. Third, in the absence of *Cd3ε* and activating *Notch1* mutations, tumor cells have decreased leukemia-initiating potential.

At the time of overt leukemia, tumor cells, in principle, have acquired the minimum numbers of transforming events. Therefore, the time taken by a single LSC to induce leukemia in secondary congenic hosts represents the time required for clonal expansion. We found that the time to leukemia for a single LSC is 36 d, consistent with results published for another genetic model of T-ALL in mice caused by PTEN deficiency (Guo et al. 2008). Interestingly, leukemia develops with a latency of 31 d in $Notch1^{tg}SCL^{tg}LMO1^{tg}$ mice. Furthermore, transplantation of 7-d-old preleukemic triple-transgenic thymocytes gives rise to leukemia in recipient mice after 3 wk, thus recapitulating the above time of tumor onset. This latency corresponds to the production of $\sim 10^{10}$ cells from a single LSC, assuming a 24-h division time, comparable with the tumor mass collected from the thymi and lymph nodes of these mice. We therefore surmise that pre-LSCs might appear during the late prenatal period, as reported for ALL in monochorionic twins (Eguchi-Ishimae et al. 2008; Hong et al. 2008). Furthermore, our observations suggest that *SCL*, *LMO1*, and *Notch1* gains of functions, together with an active pre-TCR, might be sufficient to transform

thymocytes. As a corollary, the 92-d difference in median survival times between the double $SCL^{tg}LMO1^{tg}$ model and the triple $Notch1^{tg}SCL^{tg}LMO1^{tg}$ model likely represents the time required for the occurrence of *Notch1* mutations in appropriate target cells. Our observations are consistent with the view that *SCL–LMO1* oncogenes expand the DN populations, increasing the likelihood of acquisition of *Notch1* mutations in DN4 thymocytes, thereby conferring clonal dominance to transformed cells.

Materials and methods

Mouse models

We used the previously described *pSil-TSCL* (SCL^{tg}) (Aplan et al. 1997) and *Lck-LMO1* ($LMO1^{tg}$) (McGuire et al. 1992) transgenic mouse lines (Herblot et al. 2000). Both mouse lines were backcrossed onto a C57BL6/J background for >12 generations. All mouse strains were on a C57BL6/J background: $Rag1^{-/-}$ (Jackson Laboratory), $Abb^{-/-}B2m^{-/-}$ (Taconic), *Lck-NotchIC9* ($Notch1^{tg}$) (Fowlkes and Robey 2002) (NIAID/Taconic Repository, Bethesda), and $Cd3\varepsilon^{-/-}$ kindly given by P. Hugo (Malissen et al. 1995). Mice cohorts were generated by cross-breeding, and their genotypes were verified by PCR. Transplantation assays were performed by injection of leukemic thymoma cells into unirradiated $Rag1^{-/-}$ mice or purified subpopulations of preleukemic cells into isogenic Pep3B mice. All animals were maintained in pathogen-free conditions according to institutional animal care and use guidelines. Kaplan-Meier survival and statistical analysis was performed using GraphPad Prism 4.0 software (GraphPad Software, Inc.).

Notch1 *sequencing*

Amplification of *Notch1* exons 26, 27, and 34 from cDNA of tumor cells was performed by PCR. Amplification products were sequenced in both directions. Primer sequences are shown in Supplemental Table S1. Residues are numbered according to their location in *Notch1* sequence (Ensembl sequence ENSMUSG00000026923).

Clonality analysis

Genomic DNA was obtained from digestion of leukemic cell extracts with proteinase K, followed by phenol/chloroform extraction. *Tcrb* rearrangements were determined by PCR amplification (Aplan et al. 1997).

RNA analysis

Total RNA was prepared from 10,000 sorted cells as described previously (Herblot et al. 2000) using RNeasy extraction kit (Qiagen). Primer sequences used are shown in Supplemental Table S1. Real-time quantitative PCR was done with SYBR Green detection kits (Qiagen) on a Stratagene Mx3000 apparatus (Stratagene).

Microscopy

Thymoma cells were rinsed twice in PBS (pH 7.4), fixed in 2% paraformaldehyde for 10 min at room temperature, washed, blocked, and permeabilized with 10% FBS and 0.1% NP40 in PBS (pH 7.4). Cells were then incubated with rabbit anti-NOTCH1 antibody (C20R, sc-6014R, Santa Cruz Biotechnologies) in blocking buffer for 2 h. After incubation with primary antibodies, the cells were washed three times in 1× PBS (pH 7.4) and incubated with FITC-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories) for 45 min at room temperature. The coverslips were then washed three times, mounted in Vectashield with DAPI (Vector Laboratories, Inc.), and sealed. Fluorescence was analyzed on a confocal microscope LSM 510 META (Zeiss) with a 100× objective, further magnified by a zoom of 4.

FACS analysis

Single-cell suspensions were prepared from thymi or thymoma of mice at the indicated ages. Flow cytometry analysis and cell sorting were done as described previously (Herblot et al. 2000) using Pharmingen antibodies (BD Biosciences) against CD44 (IM7), CD25 (PC61.5), CD4 (RM4-4), CD8 (53-6.7), Thy1.2 (30-H12), TCRβ (H57-597), and CD3ε (145-2C11). Bone marrow KSL population was stained as described previously (Lacombe et al. 2009). ETPs ($Lin^{-}Sca1^{+}CD25^{-}$) were analyzed by staining thymocytes with c-KIT (eBioscience, 2B8), SCA1 (BD, E13-161.7), and CD25 antibodies and excluding Lin^{+} cells stained with biotinylated antibodies against B220 (RA3-6B2), TER119 (eBioscience, TER119), CD11b (M1/70), GR1 (RB6-8C5), CD8a (53-6.7), CD3ε (145-2C11), NK1.1 (PK136), TCRβ (H57-597), TCRγδ (GL3), and CD11c (HL3). Dead cells were excluded by propidium iodide staining. FACS analysis was performed on an LSRII cytometer, and cell sorting was performed on FACSAria (BD Biosciences).

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Modeling T-cell acute lymphoblastic leukemia induced by the *SCL* and *LMO1* oncogenes

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