Combined expression of pTα and Notch3 in T cell leukemia identifies the requirement of preTCR for leukemogenesis

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Notch receptors are conserved regulators of cell fate and have been implicated in the regulation of T cell differentiation and lymphomagenesis. However, neither the generality of Notch involvement in leukemia, nor the molecules with which Notch may interact have been clarified. Recently, we showed that transgenic mice expressing the constitutively active intracellular domain of Notch3 in thymocytes and T cells developed early and aggressive T cell neoplasias. Although primarily splenic, the tumors sustained features of immature thymocytes, including expression of pTα, a defining component of the pre T cell receptor, known to be a potent signaling complex provoking thymocyte survival, proliferation, and activation. Thus, enforced expression of Notch3, which is ordinarily down-regulated as thymocytes mature, may sustain pre T cell receptor expression, causing dysregulated hyperplasia. This hypothesis has been successfully tested in this article by the observation that deletion of pTα in Notch3 transgenic mice abrogates tumor development, indicating a crucial role for pTα in T cell leukemogenesis. Parallel observations were made in humans, in that all T cell acute lymphoblastic leukemias examined showed expression of Notch3 and of the Notch target gene HES-1, as well as of pTα a and b transcripts, whereas the expression of all these genes was dramatically reduced or absent in remission. Together, these results suggest that the combined expression of Notch3 and pTα sustains T cell leukemogenesis and may represent pathognomonic molecular features of human T-ALL.

T cell acute lymphoblastic leukemia (T-ALL) is a heterogeneous set of T cell leukemias that primarily afflict children and comprise 12–15% of childhood ALL (1). Although their pathogenesis is unclear and no consistent molecular or genetic pathognomonic marker exists, it has been suggested that T-ALLs are caused by aberrant gene expression as a consequence of genetic defects. However, although many genetic lesions have been reported, they only account for a minority (<10%) of T-ALL patients (2).

Most of the aberrant gene products so far associated with T-ALL belong to cellular signal transduction pathways, including several transcription factors involved in T cell development (2), which suggests that T cell transformation might arise from altered gene products, which converge onto common differentiation pathways leading to aberrant T cell development and subsequent neoplastic transformation. A rare T-ALL-associated genetic lesion has been reported to affect the Notch1 locus. In that condition, Notch1 seems to be truncated by chromosomal translocation, and to encode a dysregulated, constitutively active intracellular domain (TAN-1) (3). Notch family proteins have the conserved capacity to control cell fate in species as diverse as flies and mammals (4). Among them, Notch1 regulates the T versus B cell fate choice in lymphocytic lineage development (5). Consistent with a causal role in the leukemogenic process, T cell neoplasia developed in mice transplanted with bone marrow cells transduced with human TAN-1 (6). In addition, Notch2 has been implicated in thymic lymphoma induced by feline leukemia virus (7). Nonetheless, the generality of Notch family member involvement in lymphoblastic leukemia and the relationships between leukemogenesis and T cell development have remained uncertain.

Recently, we generated a series of transgenic founder mouse lines that expressed the constitutively active intracellular domain (IC) of Notch3, specifically in thymocytes and their progeny T cells. With a high degree of penetrance, such mice spontaneously and early in age developed aggressive transplantable T cell neoplasias that, as for the clinical course, were reminiscent of childhood T-ALL (8).

We showed that Notch3-induced tumors provide an aberrant T cell developmental model of leukemogenesis because, although these leukemias developed primarily as splenic lymphomas, they showed consistent expression of both forms of pTα, pTα^a and pTα^b (8). This phenotype was unexpected and unusual, because pTα transcripts are not usually expressed in mature thymocytes and peripheral T cells (9). We earlier showed that Notch3 is ordinarily expressed in immature thymocytes (10), at or around a critical developmental stage, known as β-selection, that is mediated by the pre T cell receptor (preTCR). The preTCR comprises a TCRβ chain associated with signaling molecules of the cell surface CD3 complex, together with a surrogate TCRα chain termed pTα (11), for which two transcripts, pTα^a and pTα^b exist (9). As a consequence of preTCR signaling, thymocytes selectively survive and become activated to proliferate. The cell activation and proliferation events set in motion by the preTCR are not exclusive of cell differentiation, but rather provoke in parallel the acquisition of CD4 and CD8, and the initiation of TCRα gene rearrangement (11). Presumably, to prevent ongoing uncontrolled, dysregulated proliferation of thymocytes, pTα expression rapidly declines as thymocytes continue their differentiation past the "β-selection" step.

To study whether the presence of Notch3-associated dysregulation of T cell development at the preTCR stage might also

Abbreviations: T-ALL, T cell acute lymphoblastic leukemia; IC, intracellular domain; preTCR, pre T cell receptor; RT, reverse transcriptase.

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occur in human T-ALL, we have investigated the expression of Notch3 and pTα in a series of T-ALL samples. We report here that both Notch3 and pTα are abnormally expressed in all human T-ALLs investigated and that such an expression correlates with the stage of the disease, being absent in remission. The critical role of Notch3-associated expression of pTα in T cell leukemogenesis has been demonstrated here by the abrogation of lymphomagenesis in Notch3 transgenic mice in which the pTα gene was inactivated. Together, these results suggest that the combined expression of Notch3 and pTα might sustain T cell leukemogenesis and characterizes T-ALL.

Methods

Mice. The generation and typing of Notch3-IC transgenic (8) and pT$\alpha^{-/-}$ (12) mice have been described elsewhere. Double-mutant animals were obtained by breeding Notch3-IC transgenic mice with pT$\alpha^{-/-}$ mice, backcrossed for at least eight generations to C57BL/6 mice and maintained on the C57BL/6 background. Transgenic offspring were further crossed with pT$\alpha^{-/-}$ mice, and resulting Notch3-IC tg+/pT$\alpha^{-/-}$ and Notch3-IC tg+/pT$\alpha^{-/+}$ animals were used for analysis.

Flow Cytometry. Freshly isolated lymphocytes from thymus, spleen, and lymph nodes were prepared and stained as described (8) and analyzed on FACScan (Becton Dickinson) with CELL QUEST software (Becton Dickinson).

All the monoclonal antibodies used were directly conjugated to FITC or phycoerythrin. The monoclonal antibodies used were against CD4 (H-129.19), CD8a (53–6.7), and TCRβ chain (H57–597) (PharMingen); phycoerythrin- and FITC-conjugated rat and hamster IgG (PharMingen) were used as a control for immunofluorescence.

Patient Samples. Under informal consent, bone marrow and peripheral blood samples from 30 children ($\leq$15 years of age), enrolled within the AIEOP-ALL95 therapeutic protocol, were taken at diagnosis of leukemia. Diagnosis of ALL was based on morphology and on cytochemical and immunophenotypic features (13). All leukemic samples were characterized by immunophenotype. Immunophenotyping was performed on leukemic blasts isolated by standard Ficoll–Hypaque (Amersham Pharmacia) density gradient centrifugation, and cell-surface as well as intracytoplasmic antigens were detected by a panel of monoclonal antibodies either by direct or indirect immunofluorescence. The criteria for marker positivity and for the subclassification of T lineage ALL (early, intermediate, and mature T-ALL) and non-T cell ALL (common ALL, AHL-L, and preB), as well as for defining the remission stage of the disease (e.g., $<$5% of blast cells in bone marrow), were adopted as described (13).

RNA Analysis. Total RNA was isolated from thymocytes, peripheral T cells, and bone marrow in guanidine isothiocyanate and further processed for Northern blot analysis and reverse transcriptase (RT)-PCR, as described (8). PCR was performed at the annealing temperature of 58°C with the following human primers: β-actin, 5′ (5′-CTACAATGAGCTGCGTGTGG-3′) and 3′ (5′-CGGTGAGGATCTTCATGAGG-3′); Notch3 C-COOH, 5′ (5′-TTCTTAGATCTTGGGGGCCT-3′) and 3′ (5′-GGAAGAAGGAGGTCCCAGAC-3′); Notch3 TM-COOH, 5′ (5′-TGTCTTGCTGCTGGTCATTC-3′) and 3′ (5′-GGGTCTCCTCCTTGGTATCC-3′); Notch3 NH_2-TM,5′ (5′-CAACCCGGTGTACGAGAAGT-3′) and 3′ (5′-GAATGACCAGCAGCAAGACA-3′); Notch1C-COOH, 5′ (5′-CTACCTGTCAGACGTGGCCT-3′) and 3′ (5′-CGCAGAGGGTTGTATTGGTT-3′); pTα, 5′ (5′-CTGCAGCTGGGTCCTGCCTC-3′) and 3′ (5′-AGTCTCCGTGGCCGGGTGCA-3′); HES 1, 5′ (5′-TCAACACGACACCGGATAAA-3′) and 3′ (5′-GTTGGGAATGAGGAAAGCAA-3′). To quantitate expression levels of the transcripts, PCR reactions were performed in the linear exponential phase of amplification throughout 10–35 cycles, and each reaction was normalized versus the β-actin transcript internal control that was subjected to the same treatment. PCR products were analyzed by agarose gel electrophoresis followed by Southern blotting and hybridizations with specific oligomers internal to the amplified sequence. Autoradiographic bands were quantitated by scanning on Bio-Rad Imager FX PhosphorImager. Amplicon specificity was verified by sequencing on automate Perkin–Elmer ABI PRISM 377 Sequencer.

Differences in sensitivity of the different primers were monitored by adding similar amounts of template to the PCR reactions. By using this method, no significant differences in sensitivity of Notch3 primers in comparison with Notch1 or among the various primers spanning different Notch3 domains were detected.

Results

Combined Expression of Notch3 and pTα in T-ALL. Thirty cases of T-ALL were examined by RT-PCR and Northern blot for the expression of Notch3, Notch1, and both forms of pTα mRNA. Expression of Notch3 and both forms of pTα was revealed by both RT-PCR and Northern blot analysis in all cases of T-ALL investigated, irrespective of the immunophenotypic and cytogenetic subclassification of the tumor. A few cases, representative of a different class of T cell leukemia (early, intermediate, and mature T), are shown in Fig. 1*A*. Notch1 expression was also usually observed in T-ALL (Fig. 1*A*); however, unlike Notch3, Notch1 expression was not pathognomonic for T-ALL, because Notch1, but not Notch3 expression, was generally detected not only in normal peripheral blood T lymphocytes (Fig. 1*A*) but also in non-T cell leukemias (Figs. 1*B* and 2*B*).

Furthermore, Notch3 and pTα mRNAs were undetectable in normal peripheral blood T cells, whereas both were detected in normal human thymocytes, indicating that their expression in diverse T-ALL was a characteristic shared with immature thymocytes (Fig. 2*A*).

The human homolog of Notch1 may contribute to the development of a rare form of T cell lymphoblastic lymphoma, when present in a rearranged form resulting in the removal of the extracellular domain and generating a constitutively active truncated form of Notch1, TAN-1 (3, 5). No gross alterations in the size of Notch3 transcript were revealed in T-ALL samples by Northern blot analysis (Fig. 2*A*). To study whether specific domains of Notch3 were preferentially overexpressed in T-ALL samples, we performed RT-PCR of Notch3 mRNA with different primers pairs spanning different regions throughout the Notch3 transcript by using exponentially amplified PCR reactions normalized to internal β-actin transcripts as a control for quantitation. Similar ratios between the levels of each Notch3 domain in T-ALL versus the corresponding regions of the wild-type full-length thymocyte transcript were observed. Therefore, we concluded that all different domains of Notch3 are similarly represented in T-ALL (Fig. 2*B*). Furthermore, RT-PCR of Notch1 mRNA, by using primers pair spanning the region encoding the intracellular domain of Notch1, showed a similar expression in T-ALL, normal thymocytes, and normal peripheral T cells (Fig. 2*B*). Finally, the different regions of the Notch3 gene described above hybridized to a similar extent to the Notch3 mRNA band revealed by Northern blot analysis, which showed similarly represented hybridizing regions throughout the Notch3 transcript (not shown). Together, these observations suggest that full-length Notch3 transcripts are expressed in T-ALL samples.

Enhanced Expression of HES-1 in T-ALL. Constitutive activation of Notch signaling triggers a few target genes, including HES-1,

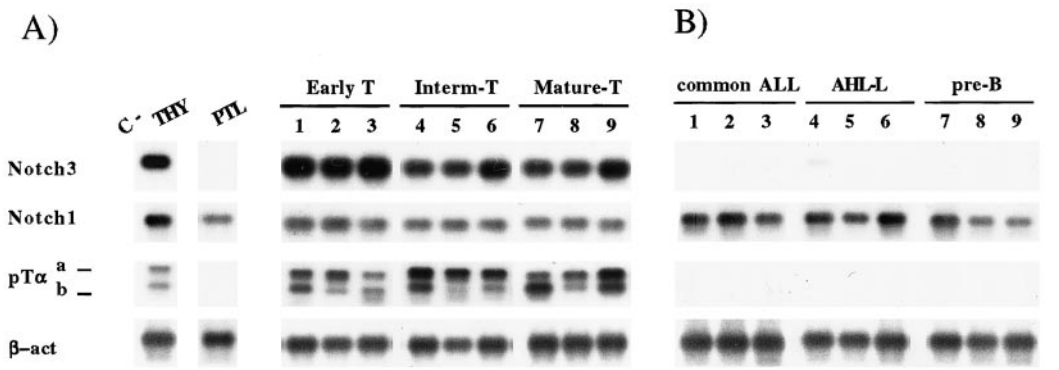

Fig. 1. (*A*) RT-PCR expression of Notch3, Notch1, and pTα a and b transcripts in early, intermediate, and mature (early-T, interm-T, and mature-T, respectively) human T cell acute lymphoblastic leukemias, classified according to ref. 13. Peripheral blood samples obtained from patients affected by T cell leukemias of different phenotypes express Notch3, Notch1, and both pTα transcripts, irrespective of the differentiation phenotype. Notch3, Notch1, and pTα expression data are representative of RT-PCR reactions monitored during the exponential phase of amplification (PCR at 25 cycles is shown) normalized to coamplified β-actin internal control to quantitate mRNA expression levels. (C-, no RNA; Thy, RNA from normal unfractionated thymocytes; PTL, RNA from enriched peripheral T lymphocytes). (*B*) Expression of Notch3, Notch1, and pTα mRNA (assessed by RT-PCR) in human non-T cell leukemias, classified according to ref. 13. Notch3 and pTα expression was measured at the end point of amplification (PCR at 35 cycles is shown), whereas Notch1 was monitored along the exponential amplification phase (PCR at 25 cycles is shown). Lack of Notch3 expression versus Notch1 did not seem to be caused by difference in sensitivity of Notch3 primers in comparison with Notch1, because similar amounts of Notch1 and Notch3 amplicons were detected starting from the addition of similar amounts of Notch1 and Notch3 templates in PCR reaction (not shown).

which is known to mediate some biological activities of the Notch pathway (14). HES-1 keeps cells in a proliferative state and plays a critical role in T cell development and regulation of CD4 gene expression (15, 16). We have reported that enhanced expression of HES-1 is also the target of the constitutive activation of Notch3 signaling in Notch3-IC-induced T cell leukemia in transgenic mice (8).

To study whether enhanced expression of a Notch target gene might also occur in human T-ALL, we examined the expression of HES-1 in T-ALL samples. We detected sustained levels of HES-1 mRNA in T-ALL, whereas it was undetectable in peripheral blood T lymphocytes (Fig. 2*C*). This result is consistent with the ability of Notch3-generated signals to regulate target gene expression.

Reduced Notch3 and pTα Expression Characterizes T-ALL Remission. To examine further the association of Notch3 and pTα expression in human T-ALL and the state of the disease, bone marrow samples at different stages of the disease were examined. The remission stage was distinguished from active disease by barely detectable levels or absence of both Notch3 and HES-1 expression, suggesting the activation of Notch3 signaling in active disease (Fig. 3). pTα expression was also decreased dramatically in remission samples, and extensive analyses showed that the most reproducible distinguishing feature was the expression of the pTα^b transcript (Fig. 3), which may represent a more sensitive indicator of the gene expression (17).

The Lymphomagenic Activity of Notch3 Requires pTα Expression. The lymphomagenic potential of Notch3 overexpression *in vivo* has

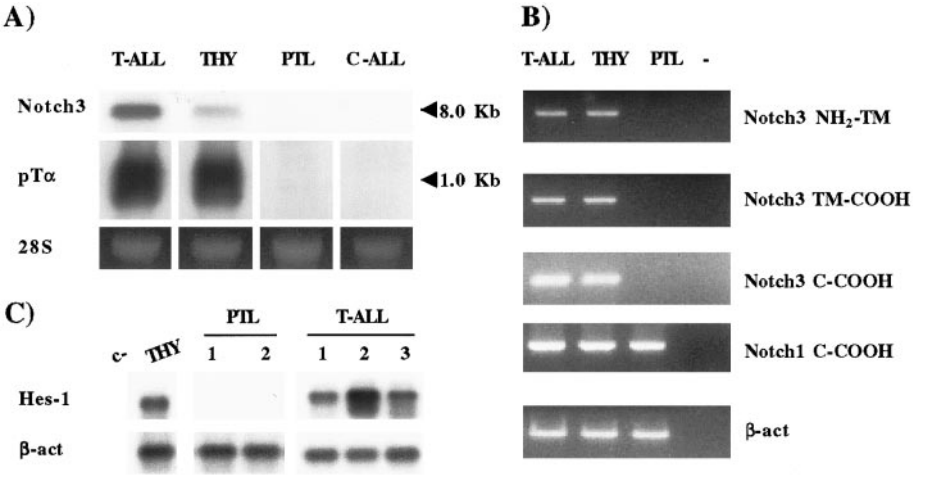

Fig. 2. (*A*) Northern Blot analysis of Notch3 and pTα mRNAs in a representative case of T-ALL, in normal unfractionated thymocytes (Thy), in enriched peripheral T lymphocytes (PTL), and in common ALL (C-ALL). (*B*) RT-PCR of Notch3 mRNA with different primers pairs spanning different regions throughout the Notch3 transcript (primers 1, C terminus, Notch3 C-COOH; 2, transmembrane, Notch3 TM-COOH; 3, N terminus, Notch3 NH_2-TM). Notch3 and Notch1 expression was monitored along the exponential amplification phase (PCR at 25 cycles is shown) and normalized to internal β-actin coamplification. Ratios of hybridization intensity between Notch3 expression in T-ALL versus thymocytes accounted for 1.1, 1.0, and 0.95, by using primers 1, 2, and 3, respectively. (*C*) RT-PCR expression of HES 1 RNA in three representative cases of T-ALL (T-ALL) and in enriched peripheral T lymphocytes (PTL) (Thy, RNA from unfractionated thymocytes; c-, no RNA). HES-1 expression was monitored along the exponential amplification phase (PCR at 25 cycles is shown) and normalized to internal β-actin coamplification.

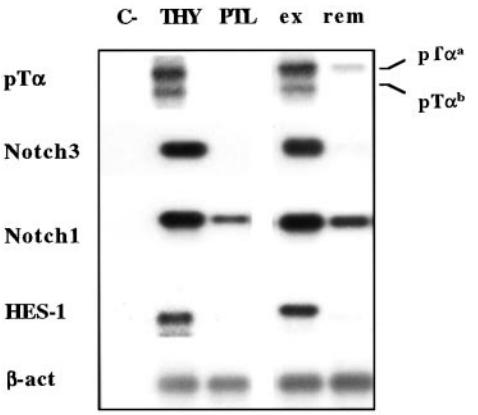

Fig. 3. Expression of Notch3, Notch1, pTα, and HES-1 mRNAs (assessed by RT-PCR) in normal thymocytes (Thy), peripheral T lymphocytes from normal subjects (PTL), and bone marrow samples from T-ALL patients (T-ALL) in different stages of human T cell leukemias (ex, exordium; rem, remission; C-, no RNA). mRNA expression was monitored during the exponential amplification phase (PCR at 25 cycles is shown) and normalized to internal β-actin coamplification.

been reported (8). The combined expression of Notch3 and pTα in human T-ALL and their absence in remission, in normal peripheral T cells and in non-T cell lymphomas, as reported here, suggests a link between Notch3 signaling pathway activation and pTα expression in human T-ALL. To investigate the relationship between Notch3 expression, pTα expression and lymphomagenesis, two lines of Notch3-IC transgenic mice were crossed to the pT$\alpha^{-/-}$ strain, and animals selected at the F2 generation that lacked both alleles of pTα. Unlike Notch3-IC/pT$\alpha^{+/+}$ mice, the follow-up of Notch3-IC/pT$\alpha^{-/-}$ mice until the age of 50 weeks showed neither splenomegaly or other overt histological or phenotypic abnormalities of lymphoid organs (Fig. 4*A*; not shown) nor early mortality in most of the mice (Fig. 4*B*), all of which are properties of Notch3-IC/pT$\alpha^{+/+}$ mice (8). Indeed, only half of the Notch3-IC/pT$\alpha^{-/-}$ mice that died spontaneously after the age of 20 weeks displayed tumor development at autopsy. Therefore, Notch3-IC/pT$\alpha^{-/-}$ mice develop tumors, albeit with a reduced kinetics, only in about 10% of cases. In contrast, the presence of a single copy of the pTα gene allows tumor development in Notch3-IC/pT$\alpha^{-/+}$. Indeed, 95% of Notch3-IC/pT$\alpha^{-/+}$ mice had died by 40 weeks and all of them displayed tumor development at autopsy, although tumor appearance and subsequent mortality were delayed with respect to Notch3-IC/pT$\alpha^{+/+}$ mice (Fig. 4*B*). These data establish that the causal role of activated Notch3 in T cell lymphomagenesis in mice depends on pTα expression.

Rescue of T Cell Development by Activated Notch3 in pTα-Deficient Mice. To study whether the absence of Notch3-induced tumors in Notch3-IC/pT$\alpha^{-/-}$ mice was due to a block of T cell development, as usually observed in pT$\alpha^{-/-}$ mice (12), we analyzed thymocyte maturation and peripheral T cell development in Notch3-IC/pT$\alpha^{-/-}$ mice versus pT$\alpha^{-/-}$ animals. Clearly, the lack of tumors in Notch3-IC/pT$\alpha^{-/-}$ mice cannot be attributed to impaired T cell development and/or a reduction in peripheral T cells. Instead, T cell development through the β-selection stage (that facilitates subsequent generation of CD4$^+$CD8$^+$ thymocytes) is rescued by Notch3 activation. We observed that the distribution of thymocyte subsets and, to same extent, the cell yield and the surface expression of TCRβ chain are rescued in Notch3-IC-pT$\alpha^{-/-}$ mice (Fig. 5 *A* and *B*). Moreover, an increased number of T lymphocytes expressing the TCRβ chain on their surface was observed in the spleen and lymph nodes of Notch3-IC/pT$\alpha^{-/-}$ mice compared with pT$\alpha^{-/-}$ (Fig. 5 *C* and *D*).

These observations suggest that activated Notch3, although able to push immature T cells through preTCR-dependent development steps in the absence of pTα, strictly requires pTα expression for sustaining its lymphomagenic potential.

Discussion

Although the Notch receptors have been reported to play a role in both T cell differentiation and T cell tumorigenesis (reviewed in refs. 18, 19), it has not been elucidated whether these processes are related to each other. In this regard, the Notch3-dependent lymphomagenesis observed in transgenic mice overexpressing a constitutively active form of this receptor seems to be a suitable experimental model (8). The Notch3-induced tumors resemble T-ALL in clinical course and phenotype and display characteristics of dysregulated differentiation such as overexpression of the pTα chain of preTCR, a feature of thymocytes at the preTCR-dependent developmental stage (8).

We report in this article that, similar to murine Notch3-induced lymphomas described (8), a combined expression of

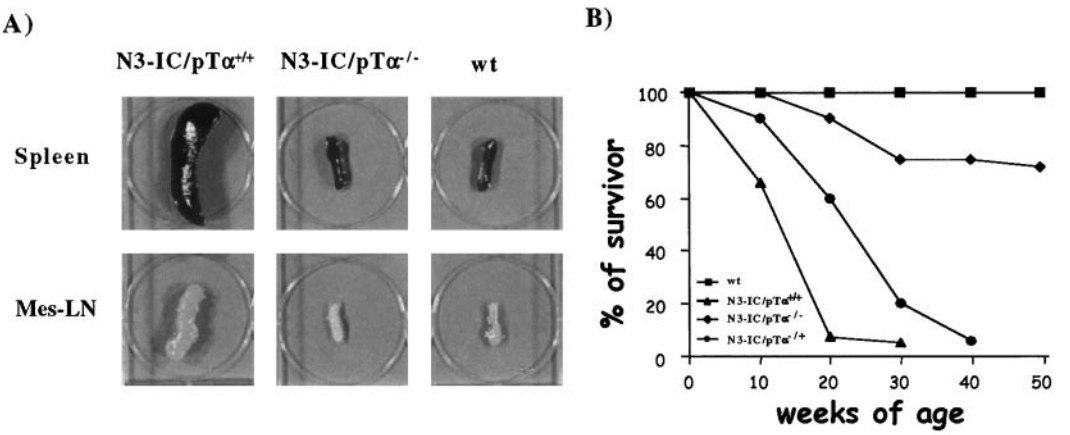

Fig. 4. (*A*) Macroscopic aspect of spleen and mesenteric lymph nodes (Mes-LN) isolated from Notch3-IC (N3-IC/pT$\alpha^{+/+}$), Notch3-IC/pT$\alpha^{-/-}$ (N3-IC/pT$\alpha^{-/-}$) transgenic and wild-type (wt) 10-week-old mice. (*B*) Mortality curve of several lines of transgenic mice. The numbers of spontaneously dead mice were plotted against their age. Results are indicated as the percentage of surviving mice at each age. The follow-up of Notch3-IC (N3-IC/pT$\alpha^{+/+}$) and of Notch3-IC/pT$\alpha^{-/+}$ (N3-IC/pT$\alpha^{-/+}$) mice was stopped at 30 and 40 weeks, respectively, being 95% of the mice dead at these ages. (*n*, 160 for N3-IC/pT$\alpha^{+/+}$; *n*, 60 for N3-IC/pT$\alpha^{-/-}$; *n*, 30 for N3-IC/pT$\alpha^{-/+}$ transgenic; and *n*, 100 for wild-type (wt) mice.

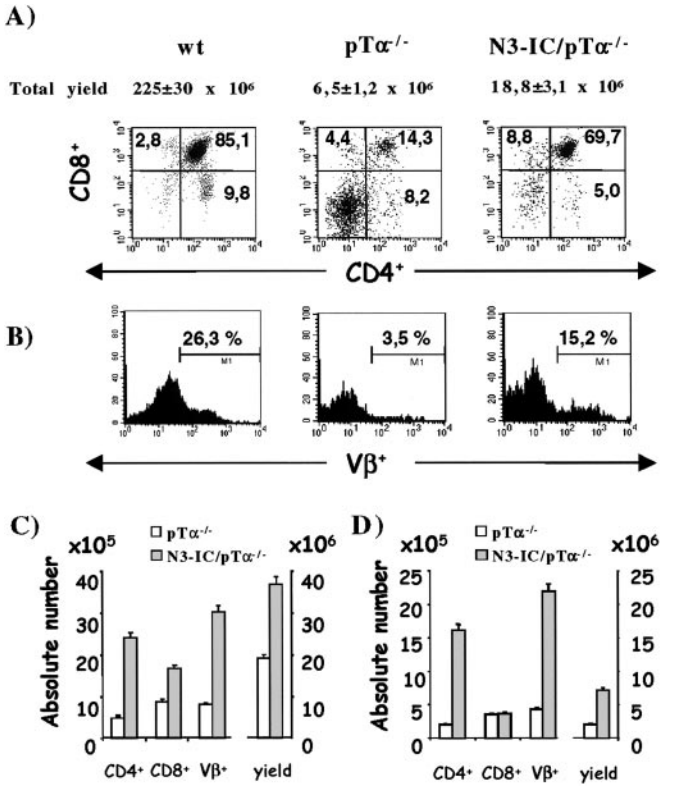

Fig. 5. Total cell yield and immunophenotype of lymphocyte from thymus, spleen, and mesenteric lymph nodes from 4-week-old mice. (*A*) Total cell yield and subset distribution of thymocytes from wild-type (wt), pTα mutant (pT$\alpha^{-/-}$), and Notch3-IC/pT$\alpha^{-/-}$ (N3-IC/pT$\alpha^{-/-}$) double-transgenic mice. Numbers in the quadrants indicate the percentage of different subsets. (*B*) Expression of TCRβ chain on thymocytes from the same mice of *A*. Numbers indicate the percentage of TCRβ high-expressing thymocytes. (*C*) Absolute number of CD4, CD8, and TCRβ chain-expressing splenic lymphocytes ($\times 10^5$) and total cell yield ($\times 10^6$) from spleen of pT$\alpha^{-/-}$ mice (pT$\alpha^{-/-}$, open bars) and Notch3-IC/pT$\alpha^{-/-}$ double-transgenic mice (N3-IC/pT$\alpha^{-/-}$, shaded bars). (*D*) Absolute number of CD4, CD8, and TCRβ chain-expressing lymphocytes ($\times 10^5$) and total cell yield ($\times 10^6$) from mesenteric lymph nodes of pT$\alpha^{-/-}$ mice (pT$\alpha^{-/-}$, open bars) and Notch3-IC/pT$\alpha^{-/-}$ double-transgenic mice (N3-IC/pT$\alpha^{-/-}$, shaded bars). Results are representative of three different experiments with animals of the same age.

both Notch3 and pTα genes characterizes human T-ALL. All human T-ALL samples examined in this study express Notch3, its target gene HES-1, and both forms of pTα mRNAs, which are neither observed in normal peripheral T cells, nor in non-T cell leukemias. Thus, these gene expression patterns distinguish T-ALL from normal peripheral blood and from non-T leukemias. Therefore, combined expression of Notch3, HES-1, and pTα seems to be pathognomonic of T-ALL. Moreover, the combined reduced expression of pTα, Notch3, and HES-1 at T-ALL remission indicates that a relationship between the Notch3 signaling pathway, pTα expression, and lymphomagenesis might exist in human T cell leukemias.

Because HES-1 is known to be a target of Notch signaling, the combined sustained expression of Notch3 and HES-1 in T-ALL leukemias is consistent with an activation of the Notch3 signaling pathway. The potential contribution of Notch1 in HES-1 regulation in T-ALL needs to be fully investigated. However, it does not seem to play a major role, because HES-1 expression apparently mainly correlates with Notch3 expression, being absent in remission and in normal peripheral T cells, despite the Notch1 expression. Moreover, enhanced expression of HES-1 has been described in lymphomas from transgenic mice expressing the constitutively active Notch3 intracellular domain (8). Although these observations are suggestive of an activated Notch3 signaling in T-ALL, specific activating signals arising from this receptor and the potential role of additional Notch proteins (e.g., Notch1) need to be elucidated. However, the up-regulation of HES-1 we observed in T-ALL samples is in keeping with the recently described potential role of this gene product in T cell lymphomagenesis, because HES-1 is constitutively overexpressed in murine T lymphoma cells carrying a murine leukemia provirus insertion in one of the Notch1 alleles, leading to a constitutively active Notch1 truncated protein (20). Transcriptional activation of HES-1 has also been shown to correlate with leukemogenesis induced in mice transplanted with Notch1 retrovirally transduced BM cells (21). Furthermore, HES-1 has been described to play a critical role in T cell development, being essential for the expansion of early T cell precursors (16). These observations, together with the data presented in this article, provide a link between the role of HES-1 in lymphomagenesis and the sustained expression of Notch3.

The nature of the relationship between Notch3 and pTα needs to be fully elucidated in T cell leukemias. Coexpression of Notch3 and pTα might reflect the development stage of the transformed cells. Indeed, both Notch3 and pTα expression characterize early stages of T cell development (10, 11). Alternatively or additionally, a causal link might exist between Notch3 and pTα expression. This scenario is suggested by the ability of Notch3-generated signals to result in increased pTα expression in thymocytes during development, as well as in mature peripheral T lymphocytes (ref. 8; unpublished results). Similarly, up-regulation of pTα by Notch1 has been reported (22), suggesting that this feature might be shared by other Notch family members. Whether such a regulation occurs at the transcriptional level needs to be elucidated. Indeed, the pTα promoter has been recently described to carry E2A- and HEB-responsive E box *cis*-elements, although their functional characterization still requires full elucidation (23–25). Nevertheless, the transcriptional function of such bHLH E proteins has been described as inhibited by Notch signaling, either by way of the Ras pathway (26) or by the Notch-target HES-1 (27). Moreover, E2A activity has been described as regulated by preTCR signaling (28). Therefore, the complexity of the multiple integrating signals regulating HLH proteins, including Notch (29), with respect to pTα transcriptional regulation, requires further thorough investigation.

Whatever the nature of the relationship between Notch3 and pTα expression is, this article highlights the essential role of pTα on T cell tumorigenesis. We demonstrated that deletion of the pTα gene from Notch3-IC transgenic mice abrogates lymphomagenesis. Furthermore, our observations suggest that the T cell lymphomagenic process is uncoupled from T cell differentiation. We demonstrated that Notch3 seems to act independently of preTCR, being able to force immature T cells to go through otherwise preTCR-dependent developmental stages and to sustain the progression toward a more differentiated phenotype by acquisition of CD4, CD8, and TCRβ expression within the thymus and in peripheral organs. In contrast, Notch3 strictly requires preTCR for sustaining its lymphomagenic potential. Such a requirement of cooperating signals generated by TCR-associated molecules has recently been reported for sustaining the lymphomagenic potential of Notch1 (30). Indeed, Notch1 retrovirally transduced hematopoietic stem cells are unable to give rise to tumors in the absence of the intracellular TCR transducer SLP-76 protein (30). However, that report does not specifically address the role of the preTCR in lymphomagenesis. Moreover, Notch1 is unable to rescue the block of T cell development in SLP-76$^{-/-}$ cells (30), whereas Notch3 is effective

in allowing generation of mature T cells from pT$\alpha^{-/-}$ progenitors (this article).

Because seemingly all cases of oncogenesis involve multiple genetic events that collaborate with each other to elicit neoplastic growth, one might hypothesize a prooncogenic role of pTα in sustaining Notch3-induced leukemogenesis. The prooncogenic potency of the preTCR may relate to its ligand independence. Indeed, the cell can be activated purely by sufficient levels of preTCR in rafts (31), suggesting a ligand-independent triggering of preTCR-dependent growth and maturation processes. Thus, the sustained coexpression of pTα and Notch3 in the periphery would be anticipated to have strong potential to activate peripheral T cells inappropriately. We hypothesize that such pathological dysregulation of proliferation and maturation processes is first manifest in early thymocytes, but that it does not preclude the cells' further differentiation and exit from the thymus. As cells continue to proliferate and to develop, their enhanced proliferative and survival potential might provide a substrate population for secondary mutations and/or further altered gene expression that would permit certain clones to grow out malignantly. The nature of those secondary mutations and/or target genes and the underlying events leading to aberrant T cell development and transformation can now be studied in T-ALL as well as in the Notch3-IC mouse model in the presence or absence of pTα gene.

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1. Pui, C.-H., Behm, F. G. & Crist, W. M. (1993) *Blood* **82,** 343–362.
2. Baer, R., Hwang, L.-Y. & Bash, R. O. (1997) *Curr. Top. Microbiol. Immunol.* **220,** 55–65.
3. Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L., Reynolds, T. C., Smith, S. D. & Sklar, J. (1991) *Cell* **66,** 649–661.
4. Artavanis-Tsakonas, S., Rand, M. D. & Lake, R. (1999) *Science* **284,** 770–776.
5. MacDonald, H. R., Wilson, A. & Radtke, F. (2001) *Trends Immunol.* **22,** 155–160.
6. Pear, W. S., Aster, J. C., Scott, M. L., Hasserjian, R. P., Soffer, B., Sklar, J. & Baltimore, D. (1996) *J. Exp. Med.* **183,** 2283–2291.
7. Rohn, J. L., Lauring, A. S., Linenberger, M. L. & Overbaugh, J. (1996) *J. Virol.* **70,** 8071–8080.
8. Bellavia, D., Campese, A. F., Alesse, E., Vacca, A., Felli, M. P., Balestri, A., Stoppacciaro, A., Tiveron, C., Tatangelo, L., Giovarelli, M., *et al*. (2000) *EMBO J.* **19,** 3337–3348.
9. Barber, D. F., Passoni, L., Wen, L., Geng, L. & Hayday, A. C. (1998) *J. Immunol.* **161,** 11–16.
10. Felli, M. P., Maroder, M., Mitsiadis, T. A., Campese, A. F., Bellavia, D., Vacca, A., Mann, R. S., Frati, L., Lendahl, U., Gulino, A. & Screpanti, I. (1999) *Int. Immunol.* **11,** 1017–1025.
11. von Boehmer, H. & Fehling, H. J. (1997) *Annu. Rev. Immunol.* **15,** 433–452.
12. Fehling, H. J., Krotkova, A., Saint-Ruf, C. & von Boehmer, H. (1995) *Nature (London)* **375,** 795–798.
13. van der Does-van den Berg, A., Bartram, C. R., Basso, G., Benoit, Y. C., Biondi, A., Debatin, K. M., Haas, O. A., Harbott, J., Kamps, W. A., Koller, U., *et al*. (1992) *Med. Pediatr. Oncol.* **20,** 497–505.
14. Artavanis-Tsakonas, S., Matsuno, K. & Fortini, M. E. (1995) *Science* **268,** 225–232.
15. Kim, H. K. & Siu, G. (1998) *Mol. Cell. Biol.* **18,** 7166–7175.
16. Tomita, K., Hattori, M., Nakamura, E., Nalcanishi, S., Minato, N. & Kageyama, R. (1999) *Genes Dev.* **13,** 1203–1210.
17. Ramiro, A. R., Navarro, M. N., Carreira, A., Carrasco, Y. R., de Yébenes, V. G., Carrillo, G., San Millàn, Rubin, B. & Toribio, M. L. (2001) *J. Immunol.* **167,** 5106–5114.
18. von Boehmer, H. (2001) *J. Exp. Med.* **194,** 43–46.
19. Rothenberg, E. V. (2001) *Nat. Immunol.* **189,** 90–92.
20. Lee, J.-S., Ishimoto, A., Honjo, T. & Yanagawa, S. (1999) *FEBS Lett.* **455,** 276–280.
21. Aster, J. C., Xu, L., Karnell, F. G., Patriub, V., Pui, J. C. & Pear, W. S. (2000*) Mol. Cell. Biol.* **20,** 7505–7515.
22. Deftos, M. L., Huang, E., Ojala, E. W., Forbush, K. A. & Bevan, M. J. (2000) *Immunity* **13,** 73–84.
23. Herblot, S., Steff, A.-M., Hugo, P., Aplan, P. D. & Trang, H. (2000) *Nat. Immunol.* **1,** 138–143.
24. Reizis, B. & Leder, P. (2001) *J. Exp. Med.* **194,** 979–990.
25. Takeuchi, A., Yamasaki, S., Takase, K., Nakatsu, F., Arase, H., Onodera, M. & Saito, T. (2001) *J. Immunol.* **167,** 2157–2163.
26. Ordentlich, P., Lin, A., Shen, C.-P., Blaumueller, C., Matsuno, K., Artavanis-Tsakonas, S. P. & Kadesch, T. (1998) *Mol. Cell. Biol.* **18,** 2230–2239.
27. Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R. & Nakanishi, S. (1992) *Genes Dev.* **6,** 2620–2634.
28. Engel, I., Johns, C., Bain, G., Rivera, R. R. & Murre, C. (2001) *J. Exp. Med.* **194,** 733–745.
29. Massari, M. E. & Murre, C. (2000) *Mol. Cell. Biol.* **20,** 429–440.
30. Allman, D., Karnell, F. G., Punt, J. A., Bokkour, S., Xu, L., Myiung, P., Koretzky, G. A., Pui, J. C., Aster, J. C. & Pear, W. S. (2001) *J. Exp. Med.* **194,** 99–106.
31. Saint-Ruf, C., Panigada, M., Azogui, O., Debey, P., von Boehmer, H. & Grassi, F. (2000) *Nature (London)* **406,** 524–527.