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*J Immunol* 2006; 177:2153-2166; doi: 10.4049/jimmunol.177.4.2153

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Structurally Distinct Ligand-Binding or Ligand-Independent Notch1 Mutants Are Leukemogenic but Affect Thymocyte Development, Apoptosis, and Metastasis Differently

Elena Priceputu, Isabelle Bouallaga, YaoPing Zhang, Xiujie Li, Pavel Chrobak, Zaher S. Hanna, Johanne Poudrier, Denis G. Kay, and Paul Jolicoeur

We previously found that provirus insertion in T cell tumors of mouse mammary tumor virus/c-myc transgenic (Tg) mice induced two forms of Notch1 mutations. Type I mutations generated two truncated molecules, one intracellular (IC) (Notch1IC) and one extracellular (Notch1EC), while in type II mutations Notch1 was deleted of its C terminus (Notch1ΔCCT). We expressed these mutants in Tg mice using the CD4 promoter. Both Notch1IC and Notch1ΔCCT, but not Notch1EC, Tg mice developed double-positive (DP) thymomas. These disseminated more frequently in Notch1ΔCCT Tg mice. Double (Notch1IC × myc) or (Notch1ΔCCT × myc) Tg mice developed thymoma with a much shorter latency than single Tg mice, providing genetic evidence of a collaboration between these two oncogenes. FACS analysis of preleukemic thymocytes did not reveal major T cell differentiation anomalies, except for a higher number of DP cells and an accumulation of TCRhighCD2highCD25high DP cells in Notch1IC, and less so in Notch1ΔCCT Tg mice. This was associated with enhanced in vivo thymocyte proliferation. However, Notch1IC, but not Notch1ΔCCT, DP thymocytes were protected against apoptosis induced in vivo by dexamethasone and anti-CD3 and in vitro by anti-CD3/CD28 Abs. This indicates that the C terminus of Notch1 and/or the conserved regulation by its ligands have a significant impact on the induced T cell phenotype. Therefore, Notch1IC and Notch1ΔCCT behave as oncogenes for T cells. Because these two Notch1 mutations are very similar to those described in some forms of human T cell leukemia, these Tg mice may represent relevant models of these human leukemias. The Journal of Immunology, 2006, 177: 2153–2166.

Members of the Notch transmembrane (TM) receptor family (Notch1–4) are broadly expressed throughout embryonic development and control cell fate decisions in many different tissues (1, 2). Notch1 has been most studied. Its extracellular domain contains 36 tandem epidermal growth factor-like repeats that function in ligand binding and three Lin/Notch repeats of unknown function. The intracellular (intra, IC) domain, which includes the RBP-Jκ-associated molecule (RAM) domain, the six cdc10/SW16/ankyrin motifs, a transcriptional-activated domain (TAD), and polyglutamine stretch (OPA), proline-glutamate-serine-threonine (PEST) sequences at its C terminus, is responsible for the signal transduction to the nucleus. Truncated forms of Notch1 representing various forms of the IC domain (Notch1IC) have been found to function as gain-of-function mutants in several biological systems.

However, studies on the effects of overexpression of Notch1IC in T cells have been controversial. Overexpression of a constitutively activated form of Notch1IC (deleted of its TAD and C-terminal PEST sequences) in transgenic (Tg) mice under the control of lck proximal promoter was initially found to promote the maturation of CD8 single-positive (SP) thymocytes (3) and to favor the TCRβ cell fate (4). In contrast, expression of another Notch1IC mutant (deleted of only its PEST sequences) was reported to favor the development of both CD4+ and CD8+ SP thymocytes in Tg mice (5). Reassessment of these two Tg strains in the same laboratory led to the conclusion that in fact both strains promote CD8+ and inhibit CD4+ SP development (6). To add to the controversy, two additional studies of Notch1IC mutants expressed in T cells of lethally irradiated mice transplanted with retrovirus-transduced bone marrow cells reported phenotypes distinct of each other and distinct from those observed in the Tg mice described above (7, 8). In one study, Notch1IC was found to prevent development of both CD4+ and CD8+ SP thymocytes (7), while in the other, it had very little impact on thymocyte development (8).

In addition to their effect on T cell development, gain-of-function mutants of Notch1IC have also been implicated in the development of T-lymphoid malignancies (9–12). TAN-1, the human homologue of Notch, was first identified as a locus involved in the t(7;9)(q34;q34.3) chromosomal translocation present in a subset of T cell acute lymphoblastic leukemias (T-ALL) (10). In these human leukemic cells, the translocation led to the aberrant expression of
of truncated Notch1 IC transcripts. Our group also found that Notch1 was mutated in a high proportion (52%) of thymic T cell lymphomas arising in Moloney murine leukemia virus-infected mouse mammary tumor virus (MMTV<sub>RI</sub>)-c-myc Tg mice (12, 13).

In these tumors, the inserted proviruses were integrated in two regions of the Notch1 gene, inducing two distinct truncated mutations: type I and II. Type I integration occurred around genomic regions coding for the 34th epidermal growth factor repeat and the TM of Notch1. Typically, type I tumors produced elevated levels of 3- to 4-kb truncated RNA transcripts, initiating at the integration site and terminating at the 3' end of the gene, and thus encoding truncated Notch1 IC proteins having conserved all IC motifs. These Notch1IC<sup>type I</sup> mutant proteins contain a small portion of extra-cellular domain, the TM domain, and the complete cytoplasmic domain (12–14). In addition, in tumor cells harboring type I mutants, truncated 5' end RNA coding for N-terminal truncated Notch1 ectodomain (Notch1<sup>EXC</sup>) was overexpressed. In type II mutants, integration of proviruses occurred within the last coding exon of the gene, thus generating a Notch1 protein deleted of its C-terminal OPA and/or PEST domains (Notch1<sup>CT</sup>). Notch1<sup>CT</sup> protein appears to be cleaved physiologically, giving rise to an intact ectodomain and a truncated Notch1 IC domain (13). Interestingly, a recent study (15) of human T-ALL cells has shown that Notch1 mutations occurred at the same two type I and type II locations as the ones generated by provirus insertions, and thus most likely generate truncated molecules very similar to those observed in murine cells harboring type I and II mutants.

Together, these results strongly suggested that truncated Notch1<sup>EXC</sup> might be involved in T cell transformation and may cooperate with c-myc in T cell oncogenesis. In fact, previous attempts to document the oncogenic potential of some truncated Notch1<sup>EXC</sup> experimentally were positive. Transduction of donor bone marrow cells expressing truncated Notch1<sup>EXC</sup> mutants led to the development of double-positive (DP) T cell malignancies in recipient mice (16). In this assay, the ankkyrin repeat and C-terminal trans activation domains were required for T cell leukemogenesis (17). Similarly, expression of two distinct Notch1<sup>CT</sup> mutants under the regulation of the <i>lck</i> proximal promotor in Tg mice was also found to induce DP T cell leukemia (3, 5, 18).

Our finding of frequent truncated Notch1 mutations in <i>myc</i> Tg mice led us to assess the oncogenic potential of these Notch1 mutants to establish a relevant animal model of the human T-ALL harboring Notch1 mutations. We chose to express the very same type I and type II Notch1 mutants initially found in murine T cell tumors in Tg mice, because they were the only ones generated and selected for during the oncogenic process. We report in this study a comparative analysis of the oncogenicity of these two mutants expressed under the same regulatory sequences of the human CD4 gene and on their different impact on T cell development.

### Materials and Methods

#### Construction of Notch1<sup>EXC</sup> Tg mice

The 14.9-kbp CD4C chimeric regulatory sequences containing the 1.9-kbp enhancer of the mouse CD4 gene and a 13-kbp fragment from the human CD4 gene have previously been described (19). The 3.0-kbp truncated Notch1 IC fragment was cleaved from mouse Notch1 cDNA (provided by H. Weintraub, the Fred Hutchinson Cancer Research Center, Seattle, WA) (20) at the NotI site (nt 5051) and at the EcoRI site (nt 8064) at the 3' end of the CD4 gene, and inserted downstream of the CD4 regulatory sequences to generate CD4/Notch1<sup>EXC</sup>. Similarly, the near-to-full-length 6.9-kbp Notch1<sup>CT</sup> DNA, deleted at the HindIII site from its 3' terminus, was inserted downstream of the CD4 regulatory sequences to generate CD4/Notch1<sup>CT</sup>.

The RI-H3 4.4-kbp Notch1 ectodomain (Notch1<sup>EC</sup>) DNA fragment and the 1.1-kbp HindIII/EcoRI C-terminal PEST-containing DNA fragment were inserted downstream of the CD4 regulatory sequences to generate CD4/Notch1<sup>EC</sup> and CD4/Notch1<sup>CT</sup> constructs, respectively. The SV40 polyadenylation sequences (0.88 kbp from plasmid NV40) (21) were ligated at the 3' end of each transgene DNA. The 18.3-kbp CD4C/Notch1<sup>EXC</sup>, 23-kbp CD4C/Notch1<sup>CT</sup>, 20-kbp CD4C/Notch1<sup>IC</sup>, and 17-kbp CD4C/Notch1<sup>CT</sup> DNAs used for microinjection were cleaved with EcoRI, then isolated by preparative agarose gel electrophoresis and further purified on CsCl gradients, as described previously (22, 23).

One-cell (C57BL/6 J C3H/HeJ) embryos were collected, microinjected, and transferred into pseudopregnant C57/BL6 females as described previously (22, 23). The procedure of the transgene was confirmed by Southern blot hybridization on tail DNA, using Notch1 CDNA as a probe. Six CD4C/Notch1<sup>IC</sup> Tg founders (F30856, F35748, F35735, F35753, and two other unnumbered founders), three CD4C/Notch1<sup>CT</sup> (F92743, F92744, F92745), four CD4C/Notch1<sup>IC</sup> (F60787, 60788, 98513, 98514), and two CD4C/Notch1<sup>EC</sup> (F87827, F87828) were produced from the pups born. All of the Tg founders were bred on the C57/BL6 background as heterozygous for the transgene for at least 8 generations and for 10–15 generations for most experiments presented. The phenotypes described in this work have been observed over a period of ~4 years without noticeable changes. All founders transmitted the transgene in a Mendelian fashion and appeared phenotypically normal. The Tg mice were observed for spontaneous thymic lymphoma development, and the thylic lymphomas were collected for analysis.

#### Analysis of transgene RNA expression

Total RNA was extracted from thymus or thymic lymphoma samples with 1 ml of TRIZol Reagent (Invitrogen Life Technologies), according to the manufacturer's protocol. To study transgene expression, RNA was separated on 1% formaldehyde-agarose gels, transferred to Hybond-N membranes (Amersham), and hybridized with Notch1 probe K for CD4C/Notch1<sup>IC</sup> Tg mice, as previously described (12). The Notch1 probe K is a 2.8-kbp BamHI/EcoRI cDNA fragment covering the entire Notch1 IC domain. Probe M was used to detect Notch1<sup>EC</sup> RNA and corresponds to a 1.9-kbp BamHI extracellular fragment of the MN7 full-length Notch1 cDNA, as previously described (12). These probes were prepared as previously described (13). For quantitation, the membrane was exposed on PhosphorImager screen and scanned with the Storm Imaging unit (Amersham Biosciences). Semiquantitation of the RNA bands was estimated with the Odyssey system and ImageQuant software (Amersham Biosciences). Semi-quantitative expression of Notch1 was calculated using 22s RNA as an endogenous control.

For analysis of c-myc Tg RNA expression, RT-PCR was performed on the RNA of thymic lymphomas arising in Notch1<sup>IC</sup>/c-myc double-Tg mice. Total RNA (5 µg) was used for cDNA synthesis with reverse transcriptase (200 U, Moloney murine leukemia virus; BRL, 200 U/µl). The cDNA obtained was used as template for PCR using primers specific for the MMTV<sub>RI</sub> long terminal repeat (LTR) (sense, 5'–GGCAAGTTTCTAA CATTACACT-3') and the c-myc exon 3 (antisense, 5'–CGGAAATGGA GATGAGCCCCGAC–5'). PCR were performed in 2.5 mM MgCl<sub>2</sub>, 1 mM dNTP, 1 U of TaqDNA polymerase, and 0.5 µM of each primer. DNA was amplified for 35 cycles at an annealing temperature of 60°C. PCR products were separated on 1% agarose gels and stained with ethidium bromide.

#### Protein extraction and Western blotting

Total proteins were extracted and immunoprecipitation assay buffer (12 mM Na<sub>H</sub>PO<sub>4</sub>, 4 mM NaH<sub>2</sub>PO<sub>4</sub>, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 10.5 mM EGTA) (24) containing 2 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin, 50 µg/ml Na<sub>p</sub>-tosyl-l-lysine chloro-methylketone, and 100 µg/ml PMSF. Extracts were cleared by centrifugation at 150,000 × g for 30 min. Protein extracts were mixed with sample buffer (62.5 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 5% β-ME), boiled for 5 min, subjected to 6% SDS-PAGE, and transferred to nylon membranes. Protein molecular mass standards (205 kDa) was purchased from Bio-Rad. Filters were blocked with 5% milk powder in TBST (10 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Tween 20) overnight at 4°C and then probed with primary antiserum in 0.5% milk powder in TBST. Immunodetection was performed essentially as described previously, using rabbit anti-Notch1-intra1 or extra1 Abs, and secondary HRP-conjugated anti-rabbit antiserum (Sigma-Aldrich 40545), followed by chemiluminescent detection (NECN). The anti-Notch1-intra1 or extra1 Abs are specific, respectively, for the Notch1 IC or extracellular domain, and their generation and characterization have been described previously (13). The resulting autoradiograms were scanned with HP Deskscan II and reproduced for publication using PowerPoint (Microsoft software). For quantitation, proteins were visualized by incubating the membranes with secondary Abs coupled to Alexa 680 fluorescein isothiocyanate (Alexa 680) followed by scanning with Odyssey infrared imaging system (Licoir). In each experiment, an image of the Notch and actin proteins was scanned on the Odyssey system and quantitated with ImageQuant software (Amersham).
Biosciences). A ratio of the Notch band over the actin band was first established. The ratio of the lower expressor founder was then set to 1 in each experiment, while the ratio of the higher expressor founder was corrected relative to 1 for each independent experiment.

**Thymocyte apoptosis in vivo and in vitro**

The CD4C/Notch1IC and CD4C/Notch1ACT Tg mice and their littermates used in this experiment were 6–12 wk old. To induce thymocyte apoptosis in vivo, dexamethasone (0.3 mg) or purified anti-CD3 (clone 145-2C11) (50 μg) was injected i.p. to Tg and non-Tg littermates, while control treatment, respectively, RPMI 1640, or purified armenian hamster IgG1 of the same isotype as anti-CD3 was injected to the control group. The mice were sacrificed after 48 or 4 h. For in vitro apoptosis, thymocytes (2 × 10^6 cells/sample) were incubated without or with dexamethasone (1 μg/ml) or anti-CD3 (25 μg/well)-coated 24-well tissue culture plates supplemented with anti-CD28 (5 μg/ml) in the medium. After 3 h, cells were harvested. The levels of in vivo or in vitro apoptosis were evaluated by labeling cells with 7-aminoactinomycin D (7-AAD) and/or annexin V/propidium iodide (PI) and anti-CD4 and anti-CD8 mAb and analysis by FACS. The number of viable cells in control mice determined the 100% viability. The relative viability in Tg or non-Tg mice was calculated by number of viable cells in treated mice compared with the number of viable cells in control mice.

**Transplantation of tumor cells into nude mice**

Thymic lymphomas from CD4C/Notch1IC or CD4C/Notch1ACT Tg mice were inoculated into nude mice. The tumors were cut into small pieces and passed through 18G needle (D = 0.3 mm) in 1 × PBS buffer. This cell suspension (~2 × 10^6 cells in 0.2 ml) was injected s.c. to 40–50 day-old CD1 nude mice. The tumor cells were injected on the right side and PBS buffer on the left side as a control.

**Flow cytometry (FACS) analysis**

Thymus, spleen, and lymph nodes (LN) from young Tg mice and their littermates, or thymic lymphomas from Tg mice were dissociated and passed through a 70-μm nylon mesh filter to obtain single-cell suspensions. To remove RBC from spleen cells, cells were subjected to lysing buffer (NH4Cl, KHCO3, EDTA (pH 7.2)) for 5 min on ice. Abs conjugated to either FITC, PE, allophycocyanin, PE-Cy5, or biotin were obtained from BD Pharmingen or Cedarlane Laboratories. Bioinylated Abs were revealed using streptavidin-TRICOLOR (Caltag Laboratories) or FITC. Thymic lymphoma cells, splenocytes, and LN cells at 1 × 10^6 cells/sample were stained for surface expression of Thy-1.2, CD2, CD3, CD5, CD44, CD25, TCR, CD4C, and mature CD8. Cells were stained in FACS buffer containing Abs and analyzed on a FACS CALIBUR apparatus (BD Biosciences). Live cells were gated according to their forward light scatter (FSC) and side light scatter profiles after staining with PI. Data were analyzed using CELLQuest software.

**Gene rearrangement analysis**

DNA was extracted from thymic lymphomas. The DNA from the kidney of the same mouse was used as control. For studying the TCRβ gene and Ig gene rearrangement, DNAs were digested with EcoRI and HindIII, respectively, and Southern blot analysis was performed with 32P-labeled probes, as described previously (25). The probe used for analyzing TCRβ gene was a 700-bp RBL-5 DNA fragment containing most of the murine C region and 3’ untranslated sequences of Cβ1. The Iγ region of Ig gene was analyzed with a 6.2-kbp EcoRI germline Iγ DNA probe.

**Histological study**

Tissues used for histology were fixed in 3.7% formaldehyde, sectioned, and stained with H&E, as described previously (26).

**Detection of cell proliferation in vivo**

This procedure has been described previously (27). Briefly, it involves the detection by FACS analysis of BrdU uptake in cell cycle and number of animals previously fed with BrdU in their drinking water (0.8 mg/ml) for 24 h or previously inoculated with BrdU (100 μg/g body weight) 4 h before sacrifice. Thymuses were collected and cell surface staining was first performed with anti-CD4 and anti-CD8 mAb, followed by an IC staining with FITC-labeled anti-BrdU Abs for detection of DNA synthesis and with 7-AAD staining for measuring total DNA content (cell cycle).

**Fetal liver transplantation**

**Donors.** Fetal livers from non-Tg and Tg 14.5-day-old embryos (E14.5) were harvested. Single-cell suspension was made in HBSS supplemented with 10% FBS, under sterile conditions, with a syringe plunger. Cell suspension was filtered through a Nytex mesh (BSH Thompson). The remaining fetal tissue was typed for Tg expression by PCR. When typing was known, cells coming from Tg or non-Tg embryos were pooled. After one wash, cells were counted (RBC cell lysis was performed in the counting aliquot). Cells were then resuspended in HBSS solution supplemented with 2% FBS at a concentration of 20 × 10^6 cells/ml.

**Hosts.** CD1 hosts (8–12 wk old) were lethally irradiated (950 rad) using Mark I-68A1 Irradiator (Cs+137; J.L. Shepherds & Associates). Hosts were injected, via the tail vein, with 4 × 10^6 fetal liver cells (FLCs) in 0.2 ml of HBSS solution supplemented with 2% FBS 4–6 h after irradiation.

**PCR technique for transgene detection**

A piece of fetal body was placed in 200 μl of lysis buffer (0.1 M NaCl, 0.01 M EDTA, 0.05 M Tris (pH 7.5), 0.5% Nonidet P-40, and 0.05% of Tween 20) supplemented with 10 μl of proteinase K (10 mg/ml) and digested at 55°C for 20 min. After digestion, lysates were centrifuged to pellet nondigested tissue, and 100 μl of supernatant was taken. This aliquot was heated at 100°C for 8 min, further diluted (10 μl in 100 μl of water), and further heated at 55°C for 30 min. PCR was performed with Tg-specific primers (sense primer, CTCTGGAAAGCACAGCGAG; product size, 300 bp). Detection of the mouse Myh gene using sense primer, CCAAGCACCTTCATCTC, and an antisense primer, GCCGTGCTTCCTCACTGCTC, was done as a control (product size, 525 bp). The PCR was performed under the following conditions (3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min at 60°C, and 1 min at 72°C, followed by 5 min at 72°C).

**Results**

Tg expression of Notch1IC induces CD4+ CD8+ thymic lymphomas

To assess the oncogenic potential of type 1 mutated truncated Notch1IC for T cells and myeloid cells, Tg mice expressing these sequences under the regulatory sequences of the human CD4 gene (CD4C) were generated (CD4C/Notch1IC) (Fig. 1A). The CD4 regulatory sequences have previously been shown to allow expression of surrogate genes in immature CD4+ CD8+ and mature CD4+ CD8+ T cells as well as in cells of the macrophage/myeloid lineage, notably in macrophages and in dendritic cells (19, 28). The Notch1IC gene contained the complete IC domain of Notch1. Of the four Tg founders harboring the CD4C/Notch1IC transgene produced and exhibiting transcript RNA expression, two died with thymic lymphoma at the age of ~1.5 mo without giving progeny. Founder lines F30856 and F35748 were established by mating the other two Tg founder mice with outbred CD1 mice. Expression of the transgene was confirmed by Northern (Fig. 1B) and by Western (Fig. 1C) blot analysis and found to be high in mice from both founder lines. As expected, expression was higher in the thymus than in the spleen (data not shown).

Groups of CD4C/Notch1IC Tg mice and their littermates were observed for the development of tumors. A high incidence of thymic lymphoma was observed: 80% (28 of 35, line F30856) and 98% (98 of 100, line F35748) of Tg mice died of, or were sacrificed with, thymic lymphoma between 30 and 180 days of age (Fig. 2A). In contrast, the non-Tg littermates from both founder lines survived beyond 9 mo without tumor development. No other signs of illness were observed at the time of death. Histopathological assessment confirmed the presence of thymic lymphomas and showed destruction of thymus architecture by atypical lymphocytes.
into 40- to 50-day-old CD1 nude mice. Of the tested thymic lymphomas, a large proportion (7 of 7, F30856; 1 of 3, F35748) grew in nude mice, reaching a tumor diameter of 1–2 cm within 30–40 days postinoculation, strongly suggesting that they were malignant.

To determine the nature of cells being transformed, TCR and Ig gene rearrangements were studied on DNA from thymic lymphomas arising in CD4C/Notch1 IC Tg mice. The TCR β-chain gene was found to be rearranged or deleted in both alleles in all nine thymic lymphomas tested (data not shown). These results indicated that these thymic lymphomas are clonal or monoclonal in origin and that they belong to the T cell lineage.

These results were further confirmed by the characterization of the transformed thymic cell population by FACS analysis. Cells isolated from thymic lymphomas were analyzed for expression of various cell surface markers of T cells (Thy-1, CD2, CD3, CD5, CD44, CD25, CD4, CD8, TCRαβ, TCRγδ, and HSA (CD24)), B cells (B220), macrophages (F4/80, Mac-1), dendritic cells (Mac-1/CD11c), and NK cells (DX-5). The lymphoma cells were distinguished from normal thymocytes by their larger size (FSC, mean channel = 142) (Fig. 2Ba). All thymic lymphomas tested (n = 22; 15 from F35748 and 7 from F30856) were of the CD4+CD8+ phenotype and were Thy-1+, CD2+. Only a few tumors among all those tested from both founders showed the presence of some SP CD4+ (2 of 22 tumors) or SP CD8+ (1 of 22 tumors) T cells. Two phenotypes could be observed according to the TCRαβ and HSA expression: type A (TCRαβhighHSA−) and type B (TCRαβlowHSA+) (Fig. 2B). Type A represented 8 of 15 and 3 of 7 tumors among those analyzed, respectively, from F35748 and F30856, the remaining belonging to type B.

The CD25 and CD44 cell surface molecules are normally expressed in CD4+CD8+ double-negative thymocytes or by activated T cells. In thymic lymphomas, DP lymphoma cells expressed high levels of CD25+ and CD44+ (12 of 15 (F35748) and 2 of 2 (F30856) tumors): F35748, SP CD25+/SP CD44+ (4 of 15), mixed SP CD25+/SP CD44+/CD25+CD25+/CD44+ (9 of 15); F30856, mixed SP CD25+/SP CD44+/CD25+CD25+CD44+ (2 of 7) and DP CD25+CD44+ (5 of 7) (Fig. 2C). However, these tumors did not express another T cell activation marker, CD69 (data not shown). The expression of CD25 and CD44, but not of CD69, in these thymic lymphoma cells suggests that these are abnormal DP cells. These DP thymic lymphomas also showed absence of B220, F4/80, Mac-1, DX-5, and CD11c staining (data not shown), ruling out an origin from B cells, macrophages, NK cells, or dendritic cells. However, in some thymic lymphomas, an increase of B220+ or Mac-1+ cells was found. This increase was not associated with any of the type A or B, nor with age.

*Tg* expression of Notch1 EC is not oncogenic for CD4+CD8+ thymocytes

Type 1 Notch1 mutation generated by provirus insertion leads to the truncation of the gene and the production of not only Notch1 IC, but also of a truncated Notch1 extracellular domain (Notch1 EC). To assess the oncogenic potential of Notch1 EC, Tg mice expressing Notch1 EC under the regulation of the CD4C sequences were constructed (CD4C/Notch1 EC) (Fig. 3A). Although these Tg mice...
expressed the transgene at high levels (Fig. 3, B–D), none of them (0 of 21) developed thymic lymphoma during the 10- to 15-mo observation period (Fig. 3E). These results indicated that Notch1IC is not oncogenic by itself for T cells.

In the tumors in which Notch1 is truncated by type I provirus insertions, we also observed that both mutated Notch1IC and Notch1EC were overexpressed. To determine whether Notch1EC might collaborate with Notch1IC for oncogenicity, double (CD4C/Notch1EC) Tg mice were generated and compared with their single-Tg littermates. The percentage of double-Tg mice developing thymic lymphoma was comparable to that of single CD4C/Notch1IC Tg mice (Fig. 3E), suggesting an absence of cooperation for T cell tumorigenicity between these two truncated molecules.

**Tg expression of Notch1ACT induces CD4+CD8+ metastatic thymic lymphomas**

Among the Notch1 mutations induced by provirus insertions, we previously described type II mutations giving rise to Notch1 molecules deleted of their C terminus (Notch1ACT). To assess the oncogenic potential of Notch1ACT, Tg mice expressing Notch1ACT under the CD4C regulatory sequences were generated (CD4C/Notch1ACT) (Fig. 4A). Three founder lines (F92743, F92744, F92745) were established by breeding with CD1 mice. Expression of the transgene was highest in the F92745 Tg mice, as determined by Northern (Fig. 4B) or Western (Fig. 4C) blot analysis. Direct comparison of expression of Notch1ACT and Notch1IC Tg RNA on the same gel with a probe common to both constructs showed that Notch1ACT from both founders (F92744, F92745) were expressed at lower levels than Notch1IC (Fig. 4B).

The CD4C/Notch1ACT Tg mice developed thymic lymphoma at various frequency in different founder lines (30%, F92743; 70%, F92744; 94%, F92745 at 10 mo of age) (Fig. 4D). In contrast to the thymic tumors of CD4C/Notch1IC Tg mice, which rarely metastasize, the CD4C/Notch1ACT thymomas were more frequently (14 of 14 total; 6 of 6, F92744; 8 of 8, F92745) accompanied by tumors in the peripheral lymphoid organs (spleen and/or LN), as observed by macroscopic pathology (14 of 14) and as confirmed by FACS analysis (8 of 10 analyzed) (see below), indicating a disseminating metastatic phenotype.

When inoculated s.c. into nude mice, most of these thymomas (5 of 5, F92744; 5 of 6, F92745) grew as local tumors of medium size (1- to 2-cm diameter) within 3–5 (F92744) or 3.5–7 (F92745) wk, indicating their malignancy.

The phenotypic characterization of the transformed thymic and peripheral cell populations done by FACS confirmed that they were DP CD4+CD8+, TCRβhigh/intermediate, CD2high, CD3high, and CD24+ or CD24− (Fig. 4, E and F), as well as CD3high (data not shown) T cells. These thymic lymphomas were negative for F4/80, Mac-1, DX-5, and CD11c staining. However, in the CD4C/Notch1IC thymoma cells, the CD4/Notch1ACT thymoma contained some B220+ and/or Mac-1+ cells. Among those analyzed (n = 6, F92744; n = 8, F92745), these tumors were positive for CD25 or for CD44 or for both (Fig. 4F): F92745, SP CD25− and CD44−; F92744, CD25− and CD44+; F92745, CD25+ and CD44−.

**FIGURE 2.** Development of thymic lymphoma in CD4C/Notch1IC Tg mice. A. Cumulative incidence of thymic lymphoma development. Data are shown for Tg mice of the F30856 (△) and the F35748 (■) lines as well as for their non-Tg littermates (○). Results are indicated as the percentage of mice that were found dead and had thymic lymphoma or were sacrificed because of dyspnea caused by thymic lymphomas. B. FACS analysis of thymic lymphomas. FSC analysis (a). Notice that thymic lymphoma cells are larger than most normal thymocytes. No difference is observed between type A and type B tumors. The numbers over the markers show the percentage of FSC. Cell surface markers on thymic lymphoma cells included CD2 (b), TCRβ (c), and HSA (CD24) (d). Data are from one non-Tg and two (#1, #3) Tg animals (F35748). The numbers in the panels show the percentage of positive cells for the respective marker and the mean fluorescence intensity. Type A (TCRβ+HSA+) and B (TCRβ−/HSA−) define two patterns of staining observed for these thymic lymphomas. C. Three-color staining with anti-CD4/anti-CD8 and anti-CD25 + anti-CD44 of thymic lymphoma cells from the same animals shown in B (#1, #3) and from one additional (#2) Tg mouse (all F35748).
Our initial screen leading to the discovery of a high frequency of provirus insertional Notch1 mutants was conducted in Tg mice. Our initial screen leading to the discovery of a high frequency of transformation process. A similar truncated Drosophila CD25 were bred on the CD1 background for at least six generations. Results for CD4C/Notch1 IC (F30856), CD4C/Notch1EC (F60787), and double (Notch1IC/Notch1EC)-Tg mice are shown. Notice the absence of thymic tumors in CD4C/Notch1 EC Tg mice. The presence of thymomas was monitored for 10 mo in Tg mice, and their non-Tg littermates were bred on the CD1 background for at least six generations. Results for CD4C/Notch1 IC (F30856), CD4C/Notch1IC (F60787), and double (Notch1IC × Notch1EC)-Tg mice are shown. Notice the absence of thymic tumors in CD4C/Notch1EC Tg mice.

(2 of 8), SP CD44+ (4 of 8), mixed SP CD25+/SP CD44+/DP CD25+/CD44+ (2 of 8); F92744, SP CD44+ (3 of 6), mixed SP CD25+/SP CD44+/DP CD25+/CD44+ (3 of 6). Together, these results indicated that the transformed cells are abnormal DP-positive thymocytes. We found only 1 tumor (of 14) with a small percentage of SP CD8+ T cells (Fig. 4F).

At the same time as the type II mutated Notch1CT is generated by provirus insertions, the Notch1 C terminus domain is found to be located downstream of the viral LTR promoter and in the same transcriptional orientation. This suggested that expression of this C terminus domain (Notch1CT) may somehow be involved in the transformation process. A similar truncated Drosophila Notch1CT domain expressed in Tg flies was found to abrogate disheveled-dependent pathways (29). To study the contribution of Notch1CT in the oncogenic process, Tg mice expressing Notch1CT under the regulation of CD4C sequences were constructed (CD4C/Notch1CT) (Fig. 4A). Two founder lines (F87827, F87828) were established by breeding with CD1 mice. Although the expression of the transgene was high, as assessed by RT-PCR analysis (data not shown), these Tg mice did not develop any thymic or peripheral tumors (0 of 32) during the 12-mo observation period, suggesting that Notch1CT is not oncogenic by itself.

**Notch1IC and Notch1ΔCT collaborate with c-myc for tumor formation**

Our initial screen leading to the discovery of a high frequency of provirus insertional Notch1 mutants was conducted in Tg mice overexpressing c-myc in DP thymocytes (MMTVD/c-myc) (12). These results not only suggested that these Notch1IC truncated forms might be involved in T cell tumor development, but also that these truncated molecules may be cooperating with c-myc for oncogenesis. To test directly whether Notch1IC is one of the c-myc cooperators in lymphomagenesis, CD4C/Notch1IC Tg mice (F30856) were crossed with MMTV D/c-myc Tg mice to generate double-Tg mice. Both Tg lines were on a CD1 background. All (100%) the double-Tg mice analyzed (n = 17) developed thymic lymphomas with a latency of 29–44 days (mean = 37 ± 4 days) (Fig. 5A). This latent period for tumor development was much shorter than that in single Notch1IC or single c-myc Tg littermates (102 ± 40 days [56.2%] and 133 ± 43 days [88.8%], respectively), within 210 days for observation. No significant difference was found in the dissemination and/or infiltration of spleen and liver between the double-Tg mice and Notch1IC or c-myc single-Tg mice bearing thymic lymphomas. Northern blot analysis or RT-PCR showed that Tg Notch1IC and c-myc were expressed in these thymic lymphomas (data no shown). Interestingly, a novel phenotype of weight loss and wasting, not present in single Notch1IC or c-myc Tg mice, was observed in some Notch1IC/myc double-Tg mice bearing thymic lymphomas. Together, these results indicated a clear collaboration of Notch1IC and c-myc in accelerating the oncogenic process in thymic T cells.

Molecular and phenotypic analysis of all thymic lymphomas (n = 17) from double Notch1IC/c-myc Tg mice showed that they...
FIGURE 4. Development of thymic lymphomas in CD4C/Notch1\(^{ACCT}\) Tg mice. A, Construct of the CD4C/Notch1\(^{ACCT}\) transgene. The human CD4C regulatory sequences (\(\square\)) were ligated upstream of the Notch1\(^{ACCT}\) sequences (\(\square\)) (aa 1–2318) containing the extracellular domain, the TM, and the IC domain deleted of its C terminus (\(\Delta CT\)). B, SV40 poly(A) signal. Bs, BssIII; E, EcoRI; H, HindIII; S, SalI; Sc, SacI. B, Northern blot analysis of Notch1\(^{ACCT}\) transgene RNA expression. Thymus (T) or spleen (S) RNA from two animals of each Tg founder line (F92743, F92744, and F92745) and from non-Tg littermates were hybridized with IC Notch1 probe K. Note the detection of the 6.9-kb Notch1\(^{ACCT}\) (N1\(^{ACCT}\)) RNA in Tg thymuses. The gel was stripped and hybridized with the actin probe to monitor gel loading. The numbers below the gel represent the intensity of the signals relative to that in the non-Tg mouse (= 1) after correction for actin intensity, as measured on the Storm PhosphorImager. For comparison of Notch1\(^{ACCT}\) expression relative to that of Notch1\(^{innos}\), the last sample of Notch1\(^{innos}\) (N1\(^{IC}\)) shown in Fig. 1B was run on the same gel as the other Notch1\(^{ACCT}\) samples. Notice the higher signal in the sample from Notch1\(^{innos}\) Tg mouse. C, Western blot analysis of Notch1\(^{ACCT}\) transgene protein expression in thymocytes. Whole-thymocyte lysates from Tg mice of two founder lines (F92744 and F92745) or from control non-Tg littermates were reacted with anti-Notch1-extra-1 Abs with Alexa 680-coupled secondary Ab, as described in Materials and Methods. The membrane was then stripped and reacted with anti-actin/Alexa 680-coupled secondary Abs. The numbers below the gel represent the relative intensity of the signals in each Tg founder line after correction for actin intensity, as measured on the Odyssey System. D, Cumulative incidence of thymic lymphoma development. Data are shown for Tg mice of the F92745 (○), F92744 (●), and F92743 (▲), as well as for their non-Tg littermates (□). These latter control mice did not develop lymphoma during this period. Results are indicated as the percentage of mice, which were found dead and had thymic lymphoma or were sacrificed because of dyspnea caused by thymic lymphomas. E and F, FACS analysis of DP cells from thymic lymphomas. FSC analysis (a), three-color staining for CD4 + CD8 and for CD2 (b), CD5 (c), TCR\(\alpha\)β (d), CD24 (e) (E), and three-color staining for CD4CD8 and CD25 + CD44 (F) were performed on thymic lymphoma cells, as described in the legend to Fig. 2, B and C. The data are represented in percentage and mean fluorescence of positive cells. Data from the same Tg (#1) or non-Tg animals are shown in E and F, while data from two additional Tg mice (#2, #3) are shown in F (all F92745). Notice that lymphoma cells are larger than most normal thymocytes. Only one immunophenotype of tumors was observed in these thymic lymphomas, similar to type B of CD4C/Notch1\(^{IC}\) Tg mice.

belong to the T cell lineage, having all a rearrangement or a deletion of the TCR\(\beta\) gene (data not shown). All the tumors analyzed by FACS (\(n = 9\)) were CD4\(^+\)CD8\(^+\) and expressed T cell-specific markers (data not shown). However, in contrast to CD4C/Notch1\(^{IC}\) or MMTV\(^{V\beta}\)c-myc single-Tg thymic lymphomas, Notch1\(^{IC}\)c-myc double-Tg thymic lymphomas could not be clearly divided into two phenotypes, and expressed different levels of TCR\(\alpha\) or HSA.

In contrast to lymphomas arising from CD4C/Notch1\(^{IC}\) single-Tg mice, a large fraction (6 of 9) of these lymphomas expressed CD44, and only a few were positive for both CD25 and CD44. None were positive for CD69 (data not shown).

To determine whether Notch1\(^{ACCT}\) could also collaborate with c-myc for tumor formation, we generated double (Notch1\(^{ACCT}\) × c-myc)-Tg mice. All (100%) these double-Tg mice analyzed (\(n = 14\)) developed thymic lymphomas with a latency of 48–80 days (mean 56 days) (Fig. 5B). The latency is much shorter than that of single Notch1\(^{ACCT}\) or single c-myc Tg littermates (mean 99 and 90 days) for the 6-mo observation period (Kaplan-Meier test, \(p <\)

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FIGURE 5. Cooperation of Notch1IC or Notch1ACT with c-myc for the development of thymic lymphoma. CD4C/Notch1IC (F30856) (A) or CD4C/Notch1ACT (F35748) (B) were crossed with MMTV1/c-myc Tg mice to obtain double Notch1IC/c-myc or Notch1ACT/c-myc (●) Tg mice and in each cross their control littermates single CD4C/Notch1IC or CD4C/Notch1ACT (■) and single MMTV1/c-myc (▲) Tg mice, as well as non-Tg mice (□). Results are indicated as the percentage of mice, which were found dead and had thymic lymphoma or were sacrificed because of dyspnea caused by thymic lymphomas. Cumulative incidence of tumors is shown.

0.0001). Six of these double Tg thymomas were further analyzed by FACS and confirmed as DP. These thymomas could be characterized as TCRαCD8high/CD2high/CD24−/CD44+/CD25− (2 of 6) or TCRαCD8high/CD2high/CD24+/CD44+/CD25− (1 of 6). Most thymomas from double-Tg mice showed dissemination/infiltration of spleen and LN (11 of 14). The immunophenotype of spleen and LN lymphomas was confirmed by FACS analysis as abnormal DP cells similar to the DP thymomas.

Preleukemic thymocytes expressing Notch1IC, but not those expressing Notch1ACT, show an increased number of abnormal (CD2high, TCRαβhigh) DP CD4+CD8+ T cells

To determine whether the expression of these gain-of-function Notch1 mutants affects T cell development, the lymphoid organs (thymus, spleen, and peripheral LNs) of young (6-wk-old) Tg mice and their control non-Tg littermates from two founder lines (F35748, F30856) were studied. A reproducible increase in the total thymocyte number was observed in the higher expressor founder (F35748) CD4C/Notch1 IC Tg mice (Fig. 6, A and B), but not in mice of the lower expressor F30856 line (data not shown).

FACS analysis showed that there was no significant difference in the FSC profiles (non-Tg = 13 ± 1.5 vs Tg = 15 ± 1.8) nor in the percentage of thymocytes staining with Thy-1, CD5, CD44, HSA, TCRγδ, and B220 or with double staining with Mac-1/CD11c CD44/CD25 between Tg mice and their non-Tg littermates (data not shown). This analysis showed a modest increase in the percentage of CD4+CD8+ thymocytes, whereas the percentage of CD4+ or CD8+ SP thymocytes was slightly decreased (data not shown). However, the increase in total cell number in mice of the F35748 line was mainly due to an increase in the DP cell population, whereas no difference could be documented for SP thymocyte subsets (Fig. 6B). Three-color analysis revealed that, in comparison with non-Tg thymocytes, a significantly higher number of these DP CD4+CD8+ Tg thymocytes from both founders were CD8high TCRαβhigh and CD25− (Fig. 6C), although changes in expression of these markers seem to occur independently from each other. These results suggest an expansion of an abnormal DP subpopulation. A similar analysis conducted on very young mice (18 days old) revealed no significant change in thymocyte numbers, nor in the percentage of the DP CD4+CD8+, SP CD4+, SP

FIGURE 6. Cell surface marker analysis of preleukemic thymocytes from CD4C/Notch1IC Tg Mice. A, FACS profile analysis was conducted on thymocytes from 6-wk-old CD4C/Notch1IC Tg mice (F35748). The thymocytes were analyzed by three-color staining with anti-CD4 and anti-CD8 plus: anti-CD2 or anti-CD25 or anti-TCRαβ. Gating was on the CD4 CD8+ DP population (R3). The numbers over the markers indicate the percentage of the indicated markers in non-Tg (thin line) and Tg (dark line) DP cells. B, Histograms show absolute cell numbers of preleukemic total thymic population (left panel) or some subpopulations, DP (left panel) and SP CD4 or CD8 (right panel) from young (6-wk-old) CD4C/Notch1IC mice (F35748). Data are from five non-Tg and six Tg mice. Data were analyzed by Student’s t test; *, p = 0.05. C, The histogram shows absolute cell number of the indicated markers (M2 for CD2 and TCRαβ and M1 for CD25) gated on DP thymocytes (R3, in A), from CD4C/Notch1IC Tg mice (F35748). Data are from five non-Tg and six Tg mice. Data were analyzed by Student’s t test; **, p < 0.01 and ***, p < 0.001.
CD8^+^, and γδ T cell subsets in Tg as compared with non-Tg mice (data not shown). In the peripheral organs (spleen and LN) of CD4C/Notch1IC Tg young mice, no significant difference was observed in the numbers and proportions of T cells (CD4, CD8) and B cells, as compared with normal non-Tg controls (data not shown).

A similar analysis conducted on the preleukemic CD4C/Notch1IC Tg mice (6 wk old) shows a small decrease of total thymocytes in mice of F92743 and F92744 Tg line (data not shown), but a very little difference in total thymocyte number in Tg mice from the high expressor F92745 line (Fig. 7B) relative to non-Tg mice. The FSC profiles of thymocytes (non-Tg = 13.5 ± 1.5 vs Tg = 14 ± 2) were comparable in Tg and non-Tg mice. The proportion and absolute cell number of DP cells were slightly decreased in Tg mice from F92743 and F92744 Tg lines (data not shown), but were also not statistically different in Tg mice from the F92745 founder line as compared with non-Tg littermates (Fig. 7, A and B). However, SP CD4 and SP CD8 thymocytes were slightly decreased only in mice from F92743 and F92744 lines (data not shown), but not in those from the F92745 line (Fig. 7B). In addition, specific changes found in CD4C/Notch1IC Tg mice were not observed. For example, we could not document a significant difference in the number of DP cells expressing TCRβ^hi^αβ^hi^ (Fig. 7, A and C). However, as in CD4C/Notch1IC Tg mice, we could document a significant increase of the CD2^hi^DP population, both in percentage (15.3 ± 8 Tg vs 7 ± 5 non-Tg) and in absolute cell number (Fig. 7C) in the three founder lines of CD4C/Notch1IC Tg mice. CD25^+^ DP cells were found to be significantly increased in percentage in all three founders (p < 0.01) (data not shown) and in absolute cell number in two (F92743, F92745) of three of the founders (Fig. 7C and data not shown). In peripheral LNs, we observed a modest increase in percentage of CD8^+, and a small decrease in CD4^+^ T cells for both founders analyzed (F92743, F92745) (data not shown). In the spleen, no significant difference could be seen.

Together, this analysis showed that expression of gain-of-function Notch1 mutants under the regulation of the CD4C sequences induces an expansion of DP cells that do not appear totally normal. This is particularly evident with the Notch1IC mutant as compared with the Notch1ACT mutant, highlighting the importance of the C terminus Notch1 domain.

Notch1IC or Notch1ACT expression leads to increased proliferation of DP CD4^+^CD8^+^ thymocytes in preleukemic mice

To determine whether the increased number of DP thymocytes of CD4C/Notch1IC Tg mice reflects an enhanced proliferation, cell cycle analysis was performed with 7-AAD on freshly isolated DP thymocytes from 6-wk-old Tg (n = 12) (F30856) and non-Tg (n = 5 non-Tg) and 11 Tg mice. Statistical analysis was done with Student’s t test; *, p < 0.02; **, p < 0.001.

**FIGURE 7.** Cell surface marker analysis of preleukemic thymocytes from CD4C/Notch1ACT Tg mice. A, FACS profile analysis of thymocytes from young (6-wk-old) CD4C/Notch1ACT Tg mice (F92745). The thymocytes were analyzed by three-color staining, as described above in the legend to Fig. 6A. B, Histograms show absolute cell number of preleukemic total thymic population (left panel) or some subpopulations, DP (left panel) SP CD4, or CD8 (right panel), from young (6-wk-old) CD4C/Notch1ACT Tg mice (F92745). Data are from 7 non-Tg and 11 Tg mice. Data were analyzed by Student’s t test and showed no statistical significance between non-Tg and Tg groups. C, The histogram shows absolute cell number of the indicated markers, as described above in the legend to Fig. 6C for cells gated on DP thymocytes (R3, A) from CD4C/Notch1ACT Tg mice (F92745). Data were pooled from 7 non-Tg and 11 Tg mice. Statistical analysis was done with Student’s t test; *, p < 0.02; **, p < 0.001.

**FIGURE 8.** Proliferation of Tg thymocytes expressing Notch1IC or Notch1ACT. A, BrdU was injected i.p. (100 μg/g body weight) and mice were sacrificed 4 h later. Thymocytes were prepared and first surface stained with anti-CD4 and anti-CD8. They were permeabilized, and their DNA was digested and stained intracellularly with anti-BrdU FITC. Data were pooled from five non-Tg, five Tg Notch1ACT (N1IC) (F35748), five Tg Notch1ACT (N1ACT) (F92744) (44), and five Tg Notch1ACT (F92745) (45) mice. The histograms show the percentage of BrdU-positive DP, SP CD4^+^, and SP CD8^+^ thymocytes from the different founder lines. Data were analyzed for statistical significance by Student’s t test. ***, p < 0.001; **, p < 0.01; *, p < 0.05.
FIGURE 9. Notch1IC, but not Notch1ICT, protects DP thymocytes against dexamethasone or anti-CD3 mAb-induced apoptosis. Apoptosis was studied in vivo 48 (A–D) or 4 h (E) after injection of the apoptotic stimuli and in vitro (F). A, Histogram presenting the total thymocyte number from Notch1IC (F30856) and Notch1ICT (F92745) Tg mice 48 h after in vivo treatment with dexamethasone or anti-CD3 mAb. Data are from 7 non-Tg and 7 Tg mice. Data were analyzed by Student’s t test; ***p < 0.001. B, FACS analysis of CD4 and CD8 expression in thymocytes from CD4C/Notch1IC (F30856, 6-wk-old) Tg and their non-Tg littermates. Animals were inoculated i.p. with dexamethasone (0.3 mg) or with anti-CD3 mAb (50 μg/mouse) or with vehicle (RPMI 1640 or hamster IgG) and sacrificed 40–48 h later. Thymocytes were stained with anti-CD4 + anti-CD8 mAb and 7-AAD. The numbers in the panels showed the percentage of viable cells (first gated as 7-AAD-negative cells) in each subset. C, Histogram presenting the absolute cell number of DP thymocytes from Notch1IC (F30856) and Notch1ICT (F92745) Tg mice. The analysis was done as described above for A. Data were pooled from four independent experiments with 7 non-Tg, 10 Notch1IC, and 8 Notch1ICT Tg mice for dexamethasone treatment and from three experiments with 3 Notch1IC Tg, 4 Notch1ICT, and 6 control non-Tg mice for anti-CD3 mAb treatment. ***p < 0.001. D, Histogram presenting the percentage of apoptotic/dead (7-AAD-positive) DP thymocytes after dexamethasone or anti-CD3 treatment. Data were pooled from the same experiments, with the same total number of animals indicated above in the legend for C. Data were analyzed for statistical significance by Student’s t test. ***p < 0.001; **p < 0.01. E, Histogram presenting the absolute cell number of DP thymocytes from Notch1IC (F30856) and Notch1ICT (F92745) Tg mice. Animals were inoculated i.p. with dexamethasone or with anti-CD3 Ab or with vehicle and sacrificed 4 h later. The analysis was done as described above in B. For anti-CD3 treatment, data were pooled from 4 non-Tg, 4 Notch1IC (F35748), 4 Notch1ICT (F92744), and 4 Notch1ICT (F92745) Tg mice. For dexamethasone treatment, data were pooled from 4 non-Tg, 4 Notch1IC (F35748), 5 Notch1ICT (F92744), and (Figure legend continues)
Notch1IC, but not Notch1ΔCT, protects thymocytes from dexamethasone or anti-CD3-induced apoptosis in vivo and in vitro

Because Notch1 insertional mutation was initially observed by our group in tumors of Tg mice overexpressing c-myc in the thymus, we speculated that these gain-of-function insertional Notch1 mutants could be involved in apoptosis. In our previous study, Tg and non-Tg control mice were inoculated i.p. with dexamethasone or anti-CD3 Abs. Forty-eight hours after the treatment, Tg mice (F30856) exhibited a higher number of their total thymocytes than non-Tg littermates (Fig. 9A). FACs analysis of thymocytes of these mice revealed a significantly (p < 0.001) higher proportion of remaining CD4+CD8+ DP thymocytes in Tg than in control non-Tg mice treated with anti-CD3 (75.98 ± 6.67% vs 18.2 ± 11%) or with dexamethasone (72 ± 8% vs 10 ± 5%) (Fig. 9B). This protection of DP cells by Notch1IC was also evident by their higher (~10-fold) numbers remaining after both treatments (Fig. 9C). Labeling with 7-AAD for detection of apoptotic/dead cells confirmed a lower proportion of dead cells among Tg DP thymocytes compared with that among non-Tg DP thymocytes, after dexamethasone or anti-CD3 treatment (Fig. 9D).

A similar experiment conducted in CD4C/Notch1ΔCT Tg mice (F92745) did not show significant protection against dexamethasone- nor anti-CD3-induced apoptosis of DP thymocytes (Fig. 9, C and D).

Because assessment of apoptosis at 48 h following the apoptotic stimuli may reflect not only the rate of apoptosis, but also the rate regenerative capacity of DP thymocytes within this interval, we performed the same experiment at 4 h following inoculation of dexamethasone and anti-CD3 mAb. The results of these experiments were very similar to those obtained after 48 h. A protection of DP thymocytes against apoptosis induced by dexamethasone and anti-CD3 mAb could be documented in CD4C/Notch1IC Tg mice (Fig. 9E). Such a protection was not evident in CD4C/Notch1ΔCT Tg relative to non-Tg mice, except for a modest protection in Tg mice of one founder (F92744). However, when compared with sham-treated animals, this group of Tg mice still had significant loss of DP cells.

To determine whether this phenotype could be reproduced in vitro, total thymocytes from control and Tg mice were incubated in vitro for 3 h in the presence or absence of the same (dexamethasone- or related (anti-CD3 + CD28 mAb) apoptotic stimuli as those used in vivo. FACs analysis was then performed by gating on DP CD4+CD8+ cells after labeling with 7-AAD to detect apoptotic/dead cells. DP thymocytes from CD4C/Notch1IC Tg mice showed a significantly lower number of 7-AAD-positive cells relative to those from non-Tg mice (Fig. 9F), suggesting that Notch1IC had a protective effect. This was not the case for DP cells from CD4C/Notch1ΔCT Tg mice, which showed comparable percentage of 7-AAD-positive cells as non-Tg ones (Fig. 9F). Similar results were obtained with annexin V/PI labeling (data not shown).

Therefore, overexpression of Notch1IC, but not Notch1ΔCT mutant protected thymocytes, especially the CD4+CD8+ DP subpopulation, from dexamethasone or TCR-induced apoptosis in vivo and in vitro. These results suggest that the C terminus domain of Notch1 may be required for this protection.

Discussion

We report in this work on the effect of the expression of two truncated Notch1 mutants, Notch1IC and Notch1ΔCT, in T cells of Tg mice.

Notch1IC mutants and T cell development

Notch1IC, but not Notch1ΔCT, was found to induce an increase of total number of thymocytes, especially of the CD4+CD8+ DP population, and to favor the appearance of a higher proportion of a CD2hi/TCRαβhi CD25+ DP subpopulation. This differential effect of these two gain-of-function mutants suggests that the mechanisms by which they impact on thymocytes may be distinct. This could possibly result from the presence of the C terminus tail

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5 Notch1ΔCT (F92745) Tg mice. Data were analyzed for statistical significance by Student’s t test. **p < 0.001; * p < 0.05. F, In vitro apoptosis. FACs profiles of DP thymocytes from CD4C/Notch1IC (N1IC) (F30856 (56) and F35748 (48)) and CD4C/Notch1ΔCT (N1ΔCT) (F92744 (44) and F92745 (45)) Tg mice 3 h after in vitro incubation with anti-CD3 + anti-CD28 mAb or dexamethasone (1 μg/ml). The thymocytes (2 × 10⁶) were harvested and stained with anti-CD4/anti-CD8 mAb and 7-AAD or annexin V/PI. Left panel, Represents the percentage of apoptotic (M1) and dead (M2) anti-CD3 + anti-CD28-treated DP cells from one representative mouse in each group. Right panel, Represents quantitation of early apoptotic DP thymocytes after gating on annexin V SP or 7-AAD SP cells (M1). Data were pooled from four experiments with CD4C/Notch1IC (F35748; n = 7); (F30856, n = 5); and CD4C/Notch1ΔCT (F92744 and F92745; n = 7 each founder for anti-CD3/CD28, and n = 9 each founder for dexa) Tg mice and control non-Tg mice (n = 8 for anti-CD3/CD28, and n = 10 for dexa). Data were analyzed for statistical significance by Student’s t test. **p < 0.001.
in Notch1IC, but not in Notch1ACT, or from the conserved regulation of Notch1ACT by its ligand(s). Unexpectedly, the other thymic or peripheral T lymphoid subpopulations were not significantly affected in their number nor in their distribution by the expression of either of the two mutants.

An enhanced expression of CD25 on Notch1IC-expressing Tg DP thymocytes has been observed previously (5, 6). However, the phenotype of the CD4C/Notch1IC Tg mice contrasts with findings in other Tg lines or in bone marrow-reconstituted mice expressing a truncated Notch1IC under the control of the lck proximal promoter (3–5) or of the LTR viral promoter (7). In the Tg mice produced by Robey’s group (3, 4), the activated Notch1IC induced an excess of CD8+ SP thymocytes relative to CD4+ SP thymocytes and favored the αβ T cell fate, but did not affect the DP cell number. In another Tg line produced by Bevan and colleagues (5), a decrease of the percentage of DP thymocytes, an increase of both CD4+ and CD8+ SP thymocytes, and a decrease of peripheral CD4+ T cells were observed. Finally, Izon et al. (7) found that expression of the intact Notch1 IC in bone marrow-reconstituted mice abrogated the appearance of SP CD4+ and CD8+ thymocytes.

The reason for the differences between the phenotypes of CD4C/Notch1IC Tg mice and those of the other Notch1IC-Tg or reconstituted strains (themselves discordant (6)) is not obvious, but may first reflect the different promoter (CD4C vs proximal lck or viral LTR) controlling Tg expression. In adult mouse thymus, the expression of the lck proximal promoter is high in immature CD4−CD8− (double-negative) and DP thymocytes, but low in more mature SP thymocytes and in peripheral T cells (30, 31). In contrast, the CD4C promoter is expressed in myeloid cells, as well as in DP and SP CD4+ thymocytes, and in peripheral CD4+ T cells, but not in SP CD8− thymocytes (19). The lack of NotchIC expression in SP CD8− thymocytes and its sustained expression in SP CD4+ T cells of CD4C/Notch1IC Tg mice may explain the absence of an expansion of the SP CD8− T cells and the lack of a block of differentiation of DP into SP thymocytes, as observed in the other Tg or reconstituted mice (3, 5, 7).

Another reason for the different phenotypes developing in CD4C/Notch1IC or lck/Notch1IC Tg lines may be related to the different domains of Notch1 expressed as a transgene. The Notch1IC construct used in this study contains a short portion of the extracellular domain, the TM domain, and an intact IC (aa 1659–2533). Kawamata et al. (8) used a retrovirus harboring an intact Notch1 IC similar to the Notch1IC studied in this work to infect bone marrow cells and reconstitute mice. They found no major changes in the distribution of DP or SP thymocyte populations, a result very similar to ours. In contrast, the transgene used by Robey et al. (3) contains only part of the IC domain, namely the ankyrin repeat region and the nuclear localization sequences (aa 1750–2293), but is deleted of the RAM domain, the TAD, and PEST sequences. The transgene used by Bevan and colleagues (5) contains the RAM, the ankyrin repeats, and the TAD domain, but lacks the C-terminal PEST domain (aa 1751–2444). These major structural differences are likely to affect the IC localization of the protein and/or its interaction with its effectors, thus possibly leading to distinct signal outputs. Already, our own data comparing Notch1IC and Notch1ACT expressed under the regulation of the same CD4C promoter strongly suggest that these two similar, but distinct truncated IC sequences affect thymocytes differently (regarding their number, their distribution, and their protection against apoptosis; see below), suggesting that the C-terminal PEST sequences are involved in this effect. The C-terminal OPA and PEST regions have been shown to bind with Numb and Disheveled proteins, both modifiers of the Notch signaling pathway (29, 32).

In an attempt to understand the cellular basis of the enhanced CD4C/Notch1IC DP thymocyte number, we found that they show a higher proliferation index, and were also more resistant to glucocorticoid and anti-CD3-mediated apoptosis in vivo, although untreated Tg and non-Tg thymocytes showed similar levels of apoptosis. This latter result is in apparent contrast with the significant increase of apoptotic thymocytes in other lck/Notch1IC Tg mice (5). Also, our results contrast with the previous work of Robey et al. (3), demonstrating that expression of Notch1IC did not promote thymocyte proliferation in vivo, because enhanced proliferation of thymocytes was clearly visible in both Notch1IC and Notch1ACT Tg mice that we studied. However, our results are consistent with other previous work showing that expression of Notch1IC could protect against DP T cell depletion in very sick mice (8), and inhibited glucocorticoid or TCR-induced apoptosis in vitro, in T lymphoma and T hybridoma cell lines, and in primary Tg T cells (33, 34), as well as arsenite-induced apoptosis in Hodgkin Reed-Steinberg cells (35). Our results also extend previous work by showing protection against apoptosis in vivo, by using an additional stimulus (anti-CD3) and an additional Notch1 mutant (Notch1ACT). Interestingly, only Notch1IC−, but not Notch1ACT, expressing DP thymocytes were protected from in vivo and in vitro induced apoptosis with the stimuli used, strongly suggesting that the structure of the Notch1 IC domain is important to achieve the proper signaling, leading to this resistance against apoptosis. Even though the apoptotic stimuli used in this study in vivo do not represent models of negative selection, Notch1IC-mediated resistance to apoptosis correlated with the increase number of TCRβ−CD4+CD25+ DP thymocyte subpopulation in these Tg mice. TCRβ−DP thymocytes have been reported to be resistant to negative selection (36–38), a process controlled by apoptosis (39). It is therefore possible that resistance to apoptosis of Notch1IC-expressing DP thymocytes is related to expression of TCRβ− DP thymocytes.

Oncogenicity of Notch1 mutants

The most apparent phenotype of the CD4C/Notch1IC and CD4C/Notch1ACT Tg mice is that both strains spontaneously developed thymic lymphomas. This result indicates that these truncated mutant forms of Notch1 can behave as oncogenes in immature DP cells and that the C-terminal PEST sequences are dispensable for oncogenicity, thus confirming earlier work with similar, although not identical, Notch1 IC molecules (3, 5, 6, 16, 17). All of the tested thymic lymphomas from both strains belong to the T cell lineage, being composed of CD4+CD8− T cells. These results indicate that each of those truncated forms of Notch1 is efficient at transforming some (DP thymocytes), but not all (SP CD4+ thymocytes, peripheral CD4+ T cells, macrophages) target cells in which they are expressed. The fact that both Notch1IC and Notch1ACT induced DP thymomas, while only Notch1IC was able to protect DP cells from apoptosis, provides genetic evidence that resistance to apoptosis, at least that induced by dexamethasone and anti-CD3, is not essential for the malignant transformation. This differential protection against apoptosis provided by Notch1IC, but not by Notch1ACT, also indicates that these molecules affect signaling in DP cells differently.

The observation that discrete TCR β-chain DNA fragments could be detected in these thymic lymphoma cells is indicative that these tumors are monoclonal or oligoclonal. Their oligoclonality as well as their long latency development suggest that each of these truncated Notch1 mutants itself is not sufficient to fully transform DP cells. Additional events (second-hit) are required for tumorigenesis. Our previous studies already suggested that Notch1 is a cooperator of c-myec for T cell transformation, because truncation
of Notch1 by provirus insertion was a very frequent event (~60%) in T cell tumors arising in Moloney murine leukemia virus-infected myc Tg mice (12). The results of the experiments with double (CD4/Notch1IC × MMTVp10c-myc) or (CD4/Notch1IC × MMTVp10c-myc) Tg mice presented in this work provide direct evidence that overexpression of c-myc can represent such a second-hit event cooperating with Notch1 in T cell lymphomagenesis. The obvious collaboration of Notch1IC and c-myc for tumor formation, in absence of protection of Notch1IC against dexamethasone or anti-CD3 mAb-induced apoptosis, suggests that the resistance to apoptosis per se does not appear to be an essential element of this c-myc collaboration. The molecular basis of this synergy remains to be elucidated. A similar Notch1IC and c-myc quantitation analysis.

Moreover, neither of these mutants can provide a preferential expansion of SP CD8 T cells, and distribution, as well as their response to apoptotic stimuli. The direct comparison of two gain-of-function mutants of Notch1 for oncogenesis.

Conclusion

The direct comparison of two gain-of-function mutants of Notch1 expressed with the same regulatory elements (CD4C) in T cells of Tg mice shows that they differentially affect DP thymocyte number and distribution, as well as their response to apoptotic stimuli. Moreover, neither of these mutants can provide a preferential expansion of SP CD8+ or y6 thymocytes or induce a differentiation block of DP into SP thymocytes, as previously described in other Tg mice expressing truncated Notch1 IC sequences of a different structure under the regulation of other promoters. Both mutants are oncogenic for DP thymocytes, but expression of Notch1IC strongly favors metastasis. Together these results suggest that the structure of the gain-of-function mutants of Notch1, as well as the subset of T cells in which they are expressed have a very significant impact on the induced T cell phenotype. These variables may be critical for the generation of relevant animal models for human T-lymphoblastic leukemia harboring Notch1 mutations. Interestingly, it was recently reported (15) that these human mutations are almost identical with the type I and II murome Notch1 mutants expressed previously by our group (12, 13). These are precisely the mutations that we have attempted to partly mimic in this study by expressing Notch1IC and Notch1IC.

Acknowledgments

We thank Ginette Massé, Benoît Laganière, Michel Robillard, Karina Lamarre, Pascale Jover, Valérie Côté, and Jean-René Sylvestre for their excellent technical assistance. We are grateful to Marc Cour for his help in quantitation analysis.

Disclosures

The authors have no financial conflict of interest.

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