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High Incidence of T-Cell Lymphomas in Mice Deficient in the Retinoid-related Orphan Receptor RORγ[1]

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ABSTRACT

Nuclear receptors are critical regulators of many physiological processes and have been shown to be involved in a variety of disease processes, including malignant neoplasms. Our laboratory is investigating the function of the retinoid-related orphan receptor γ (RORγ) and its possible role in disease. Studies of mice deficient in the expression of RORγ demonstrated that this receptor plays a crucial role in the regulation of thymopoiesis and lymph node organogenesis. In this study, we show that changes in homeostasis in the thymus of RORγ$^{-/-}$ mice are associated with a high incidence of T-cell lymphomas. Over 50% of the deficient mice of mixed genetic background die within the first 4 months as a result of thymic lymphomas. A high incidence of lymphomas was also observed in RORγ$^{-/-}$ 129/SvEv mice. The lymphoblastic cells metastasized frequently to spleen and liver. No other tumor types were detected in any of RORγ$^{-/-}$ mice that died during the course of the experiment, and none of the heterozygous mice developed thymic lymphomas. Lymphoma formation was associated with increased cellular proliferation and an increase in the number of apoptotic cells. When placed in culture, the RORγ$^{-/-}$ lymphoblastic cells underwent accelerated "spontaneous" apoptosis at a rate similar to that of RORγ$^{-/-}$ thymocytes. Upon prolonged culture, several lymphoblastic cell lines could be established. Analysis of the immunophenotype of the lymphoblastic cells showed that the CD4 and CD8 subpopulations varied substantially among different lymphomas. The established cell lines consisted mostly of CD44^{-}CD25^{+}CD4^{-}CD8^{-} cells. Our studies indicate that loss of RORγ disturbs homeostasis in the thymus by enhancing apoptosis and cellular proliferation. The latter may enhance the probability of individual cells to acquire genetic alterations that make them escape negative selection and normal differentiation programs and as a consequence lead to increased susceptibility to the development of T-cell lymphoma.

INTRODUCTION

Nuclear receptors constitute a family of ligand-dependent transcription factors that regulate a broad spectrum of physiological processes, including embryonic development, apoptosis, cellular differentiation, and proliferation (1–3). Because of all of these processes, their role in the etiology of malignant transformation and their potential as targets for chemoprevention and treatment of cancer have been explored extensively. For example, chromosomal translocations involving the retinoic acid receptor RARα have been implicated in promyelocytic leukemia and retinoids have been successfully used in chemotherapy of this cancer (4, 5). Similarly, the role of the estrogen receptor and its ligands in the development, chemoprevention, and treatment of breast cancer is well documented (6). Less, however, is known about the role of many other nuclear receptors, including the RORs.[3]

RORα, RORβ, and RORγ (named NR1F1 to NR1F3 by the Nuclear Receptor Nomenclature Committee) belong to a subfamily of nuclear orphan receptors and have been reported to regulate a number of diverse physiological processes (7–12). RORα has been shown to control differentiation of Purkinje cells, and defects in Purkinje cell development have been linked to the ataxia phenotype exhibited by RORα$^{-/-}$ mice (13–15). In addition, RORα$^{-/-}$ mice placed on a high fat diet have an increased risk developing atherosclerosis (16). Different functions have been identified for RORβ. RORβ is most highly expressed in tissues involved in processing sensory information and in anatomical components implicated in the regulation of circadian rhythm (17), suggesting a role for RORβ in the control of these processes. The latter is supported by the observed abnormalities in circadian behavior in RORβ$^{-/-}$ mice (17, 18).

The RORγ gene generates two isoforms referred to as RORγ1 and RORγ2 (also named RORγt; Refs. 12, 19–21), which differ only in their NH_2 terminus. The two isoforms are derived by transcription from alternative promoters and exhibit a different pattern of expression. RORγ1 is expressed in several tissues, including liver, thymus, kidney, and brown fat cells, whereas RORγ2 is restricted to the thymus, where it is expressed mostly in "double-positive" (CD4^{+}CD8^{+}) thymocyte subpopulations with some expression in early precursor CD45^{+}CD25^{-} cells (10, 19, 22–24). Recently, we and others (25, 26) reported that mice deficient in RORγ lack lymph nodes and Peyer's patches, indicating that RORγ is essential in lymph node development. In addition, thymocytes from RORγ$^{-/-}$ mice undergo accelerated apoptosis that is related, at least in part, to a down-regulation of the expression of the antiapoptotic gene *Bcl-*X_L (25, 26). These findings suggest an important role for RORγ in the regulation of homeostasis in the thymus. To examine the long-term effects of RORγ deficiency, a large group of RORγ$^{-/-}$, RORγ$^{+/-}$, and wild-type littermates were selected and monitored for a 1-year period. In this study, we demonstrate that RORγ$^{-/-}$ mice are highly susceptible to early onset of thymic lymphoma. Analyses of the immunophenotype and the rate of apoptosis and proliferation of the lymphocyte populations indicate that the lack of RORγ expression results in a dysregulation of proliferation and apoptosis, thereby disturbing normal homeostasis. This dysregulation may enhance the probability of individual cells to acquire mutations and as a consequence lead to a high incidence of T-cell lymphoma development.

MATERIALS AND METHODS

RORγ-deficient Mice. The generation and initial characterization of RORγ$^{-/-}$ mice was described previously (25). The original mice were in a 129/SvEv × B6D2 × C57BL/6 background. Mice were maintained on a 129/SvEv background by crossing chimeras with 129/SvEv mice (Taconic). The p53$^{-/-}$ mice were described previously (27). RORγ$^{-/-}$ mice showing terminal signs of possible lymphoma development (*e.g.*, respiratory disturbance) were euthanized and subjected to necropsy.

Immunohistochemical Staining. Tissues were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Paraffin-embedded sec-

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[3] The abbreviations used are: ROR, retinoid-related orphan receptor; PCNA, proliferative cell nuclear antigen; RT-PCR, reverse transcription-PCR; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; CDK, cyclin-dependent kinase; TCR, T-cell receptor.

tions were rehydrated and then treated with anti-PCNA antibody (1:100; Biogenex) and subsequently with biotin-conjugated goat antimouse IgM. Biotin was visualized using Vectastain kit (Vector Laboratories).

Northern Blot Analysis. RNA was isolated from thymocytes of wild-type and RORγ$^{-/-}$ mice using Tri-Reagent (Sigma Chemical Co.) according to the manufacturer's protocol. Total RNA (30 μg) was separated by electrophoresis on a formaldehyde 1.2% agarose gel, blotted to an Immobilon-Ny^{+} membrane, UV-cross-linked, and then hybridized to various ^{32}P-labeled RORγ, notch-1, p53, c-myc, and Bcl-X_L probes. Hybridizations were performed at 68°C for 1 h, the membranes were then washed twice with 2× SSC, 0.1% SDS at room temperature for 30 min, and 0.1% SSC, 0.1% SDS at 60°C for 30 min. Autoradiography was carried out with Hyperfilm-MP (Amersham) at −70°C.

Cells and Tissue Culture. Thymocytes, splenocytes, and peripheral blood cells from 8-week-old mice were isolated as described (25) and used immediately for analysis. Thymocytes (1–2 × 10^6 cells/ml) were also cultured for various times in RPMI 1640 containing 10% FBS.

Flow Cytometric Analysis. Single cell suspensions were incubated with a combination of Cy-Chrome-, FITC-, or phycoerythrin-conjugated CD4, CD3, CD44, CD25, and CD8 antibodies (PharMingen) as described previously (25). For cell cycle analysis, cells were fixed in 70% ethanol and resuspended in PBS containing propidium iodide (50 μg/ml). Analysis of FITC-Annexin V binding was carried out using the ApoAlert Annexin V kit (Clontech) according to the manufacturer's instructions. Flow cytometric analyses of labeled cells were performed with a FACSort (Becton Dickinson) or EpicsXL (Beckman-Coulter) flow cytometer and accompanying FACS Convert, CellQuest, and MacCycle (Phoenix Flow Systems) software programs.

RT-PCR. cDNA were prepared from total RNA of RORγ knockout and wild-type mice thymocytes using Moloney murine leukemia virus reverse transcriptase (Stratagene) with random primers. The BRCA2-specific primers were 5′-GGTCTTGCTCCTTTGGTCTA and 5′-GCCTCCTTGTTAAATGTATC. PCR was performed with 34 cycles consisting of first denaturing at 94°C for 5 min and 34 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a 10-min extension at 72°C for the last cycle; β-actin mRNA was amplified as an internal control. The RT-PCR products were then separated on a 1% agarose gel and visualized with staining by ethidium bromide.

TUNEL Staining. For detection of DNA fragmentation *in situ*, paraffin-embedded sections were analyzed by the TUNEL method using an *In Situ* Cell Death Detection kit (Roche) according to the manufacturer's protocol. Labeling by TMR red-labeled dUTP was examined in a ZEISS Axioplan fluorescence microscope.

Statistical Analysis. The Kaplan-Meier test was used to calculate the latency to tumor formation, and the log-rank test was used for evaluation of significance. The significance of differences in frequencies between groups and/or tumor types was evaluated by χ^2 analysis. $P < 0.05$ was considered as significant.

RESULTS

Previous studies of RORγ$^{-/-}$ mice have demonstrated that RORγ plays a crucial role in the control of thymopoiesis and lymph node development (25, 26). To examine the effect of this phenotype on the long-term health status, a large group of RORγ$^{-/-}$, RORγ$^{+/-}$, and wild-type littermates were selected, and their health status was monitored and compared over a 1-year period. As expected, the majority of the wild-type animals remained healthy, and no tumors were identified on necropsy of the two wild-type animals that died during this time period (Fig. 1*A*). Heterozygosity for the mutant *RORγ* allele did not result in a significant change in survival over 1 year. Four of the 38 heterozygous mice died during the first year of life and, although the cause of death was not established, no tumors were observed on necropsy of these animals. In contrast, by 122 days of age, 50% of the RORγ$^{-/-}$ mice had become moribund or died (Fig. 1*A*). Necropsy of 17 moribund RORγ$^{-/-}$ mice revealed the presence of a thymic lymphoma in all but 1 of these animals. The cause of death of the last mouse was not established. A small percentage of RORγ$^{-/-}$ mice, 3 of the original 32 (9.4%), were alive at the end of the 1 year experiment. No tumors were observed on necropsy of these

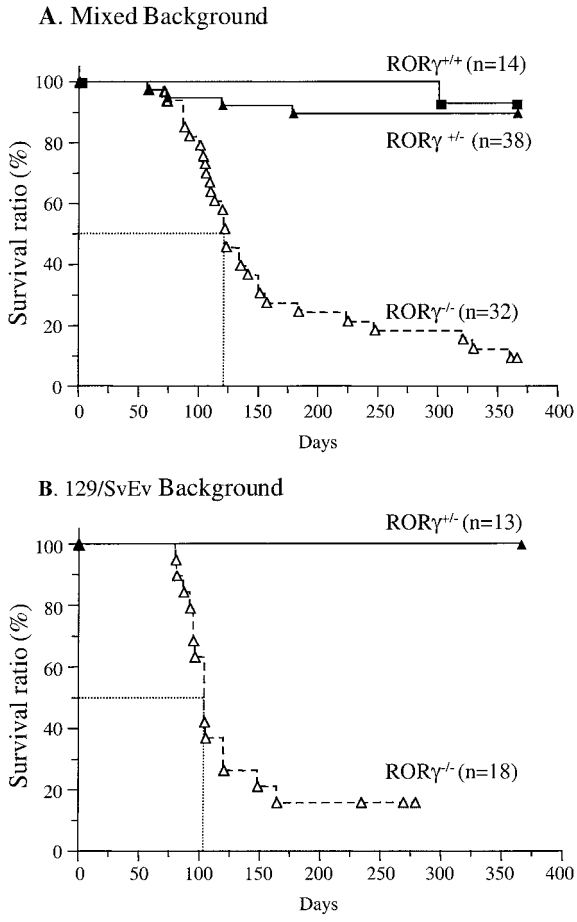

Fig. 1. Survival curves of RORγ$^{-/-}$, RORγ$^{+/-}$, and wild-type mice. *A*, survival of mice of mixed genetic background (C57BL/6 × 129/SvEv × B6D2). The percentage of mice surviving *versus* the mice alive at the onset is plotted against age. The *vertical line* indicates the time point by which 50% of the mice survived. Median survival RORγ$^{-/-}$ *versus* RORγ$^{+/+}$ mice: $P < 0.0001$; $\chi^2 = 26.10$; median survival RORγ$^{-/-}$ *versus* RORγ$^{+/-}$ mice: $P < 0.0001$; $\chi^2 = 49.33$. *B*, survival of 129/SvEv mice. Median survival RORγ$^{-/-}$ *versus* RORγ$^{+/-}$ mice: $P < 0.0001$; $\chi^2 = 27.62$. The numbers of mice used in each experiment are indicated on the *right*.

animals. In fact, the thymi of these mice were slightly smaller than age-matched wild-type mice and had reduced numbers of double-positive CD4^{+}CD8^{+} cells, as was reported for thymocytes from 8-week-old RORγ$^{-/-}$ mice (25, 26). Because no significant difference in median survival was observed between RORγ$^{+/-}$ *versus* RORγ$^{+/+}$ mice within the period of observation, these results suggest that there appears to be no gene dosage effect on survival as a result of loss of one or two alleles of RORγ. The extended survival of a small percentage of the RORγ$^{-/-}$ mice might reflect the impact of modifier genes on the development of thymic lymphomas. Because this initial cohort of animals were of mixed genetic background, it is possible that a small number of the RORγ$^{-/-}$ mice carried a combination of modifier allele(s) that protected them from the development of thymic tumors. To analyze the latter possibility, we backcrossed the RORγ$^{-/-}$ chimeras into 129/SvEv mice. Eighteen RORγ$^{-/-}$ and 13 RORγ$^{+/-}$ 129/SvEv mice were included in the study. Survival of the RORγ$^{-/-}$ 129/SvEv mice did not differ significantly from that observed for RORγ$^{-/-}$ mice of mixed genetic background (Fig. 1*B*). Fifty % of the RORγ$^{-/-}$ 129/SvEv mice were moribund or had died by 104 days of age. Necropsy of moribund mice revealed thymic lymphomas similar to those observed in RORγ$^{-/-}$ mice of mixed genetic background. There was no significant difference in the median survival between RORγ$^{-/-}$ mice of either 129/SvEv or mixed genetic

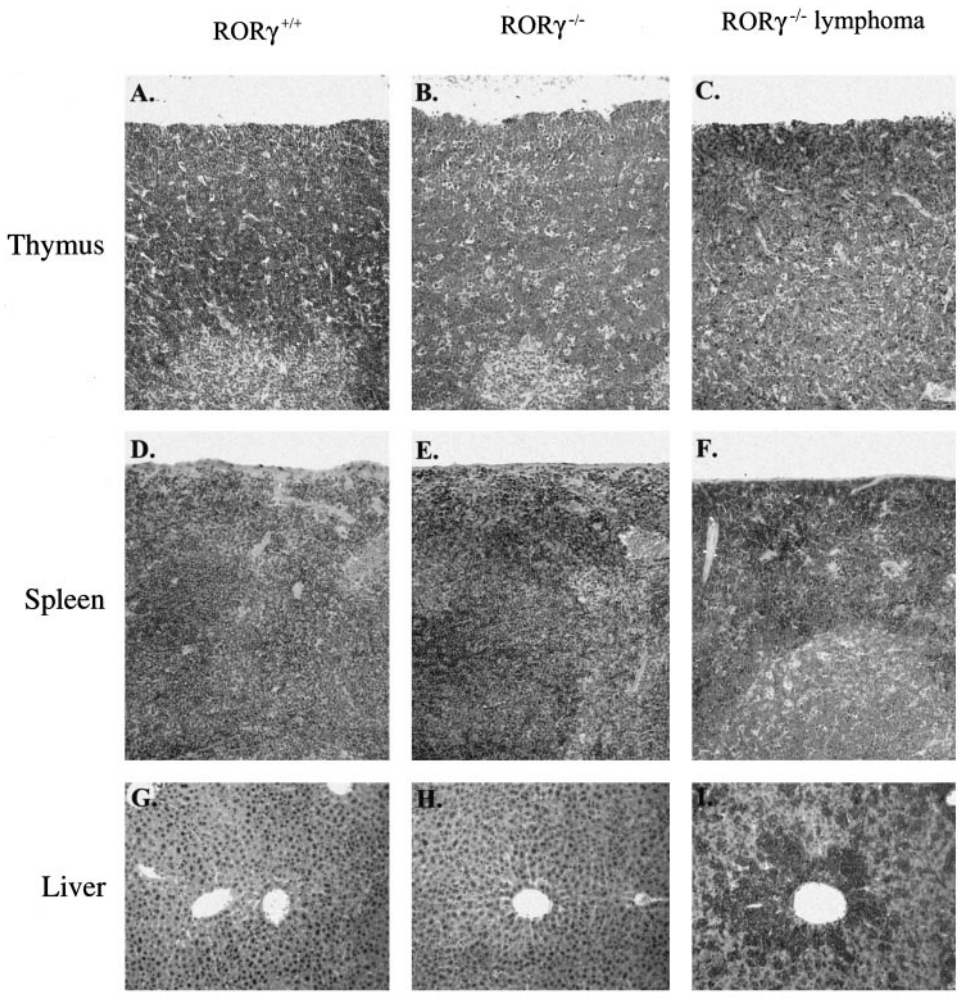

Fig. 2. *A–C,* H&E-stained sections of thymi from wild-type mice, ROR$\gamma^{-/-}$ mice without thymic tumor, and ROR$\gamma^{-/-}$ mice with thymic lymphoma. Tumors often lacked the cortical-medullary organization of the normal thymus and contained rather uniform lymphoblastic-type cells. *D–F,* H&E-stained sections of spleens from wild-type mice, ROR$\gamma^{-/-}$ mice without thymic tumor, and ROR$\gamma^{-/-}$ mice with thymic lymphoma. Although splenic architecture remained largely intact, infiltration of lymphoblastic cells can be observed in both white and red pulp regions. *G–I,* H&E-stained sections of livers from wild-type mice, ROR$\gamma^{-/-}$ mice without and with a thymic lymphoma. In *I,* massive lymphoblast infiltration can be observed in perivascular stroma in liver from ROR$\gamma^{-/-}$ mice with thymic lymphoma. The results shown are for sections obtained from mice in mixed genetic background. Similar results were obtained from mice in 129/SvEv background. ×20.

background ($P = 0.2628$; $\chi^2 = 1.254$). No deaths were observed in the ROR$\gamma^{+/-}$ animals, and no tumors were observed in these animals. However, despite the fact that the study was carried out with congenic lines, a small percentage of the ROR$\gamma^{-/-}$ mice, 2 of the 18 (11.1%) included in the study remained tumor free. These results may suggest a possible role for a stochastic element in the development of thymic lymphomas in mice of both 129/SvEv and mixed genetic background.

Although ROR$\gamma^{-/-}$ mice rapidly developed T-cell lymphomas, there was no evidence for the formation of any other tumor type in other organs, even in the few ROR$\gamma^{-/-}$ mice that survive after 1 year. In all cases, the thymic tumors in ROR$\gamma^{-/-}$ mice often had a weight 10 times that of the thymus from wild-type mice [872.6 ± 90.8 mg ($n = 11$) *versus* 62.4 ± 13 mg in wild-type]. Histological examination of the grossly enlarged thymus showed that these tumors often lacked the cortical-medullary organization of the normal thymus and contained rather uniform lymphoblastic-type cells (Fig. 2, *A–C*). The lymphoblastic cells metastasized frequently to the spleen and liver. Spleen mass in ROR$\gamma^{-/-}$ mice frequently was six times that in control mice [724.3 ± 229.6 mg ($n = 11$) *versus* 122.5 ± 16 mg in wild-type]. Although the splenic architecture remained largely intact, massive lymphoblast infiltration was observed in both the red and white pulp (Fig. 2, *D–F*). In liver sections, massive lymphoblast infiltration was observed into the perivascular stroma, as often seen in metastatic lymphoma (Fig. 2, *G–I*). Occasionally, metastasis to kidney was observed (not shown). In bone marrow, normal hematopoietic cells were replaced by a monotonous population of blastic cells with numerous mitotic and apoptotic figures, consistent with lymphoblast infiltration (not shown). Large lymphoblastic cells ($3.92 \pm 1.02 \times 10^3$ cells/ml; $n = 6$) were also observed in peripheral blood from ROR$\gamma^{-/-}$ mice bearing thymic lymphomas. Because ROR$\gamma^{-/-}$ mice do not contain lymph nodes (25, 26), no metastasis to lymph nodes was observed.

To examine the proliferative activity of cells in lymphomas, tumor sections from ROR$\gamma^{-/-}$ mice were stained for PCNA. This analysis revealed that the number of PCNA-stained cells was greatly enhanced compared with sections from wild-type thymus (Fig. 3, *A, C,* and *D*). As shown in Fig. 3*B,* PCNA staining was also enhanced in sections of thymus from ROR$\gamma^{-/-}$ mice. Thymocytes in the inner subcapsular region stained most strongly for PCNA, whereas cells in the outer subcapsular region stained only weakly. These results suggest that the number of actively proliferating cells in both ROR$\gamma^{-/-}$ lymphomas and thymi from ROR$\gamma^{-/-}$ mice is significantly enhanced. The increase in proliferative activity was confirmed by cell cycle analysis using flow cytometry (Fig. 4). This analysis showed that ~32% of the cells in lymphomas were in the S-phase of the cell cycle compared with only 4.7% of wild-type thymocytes (Table 1). It is important to point out that the percentage of thymocytes in the S-phase of the cell cycle is already significantly increased before mice develop thymic lymphomas (Table 1; Refs. 25, 26).

Previous studies showed enhanced apoptosis in thymi from ROR$\gamma^{-/-}$ mice compared with those from wild-type mice (25, 26). Examination of sections of several ROR$\gamma^{-/-}$ lymphomas by TUNEL staining showed that although the degree of apoptosis varied among tumors, tumors consistently contained a significantly higher number

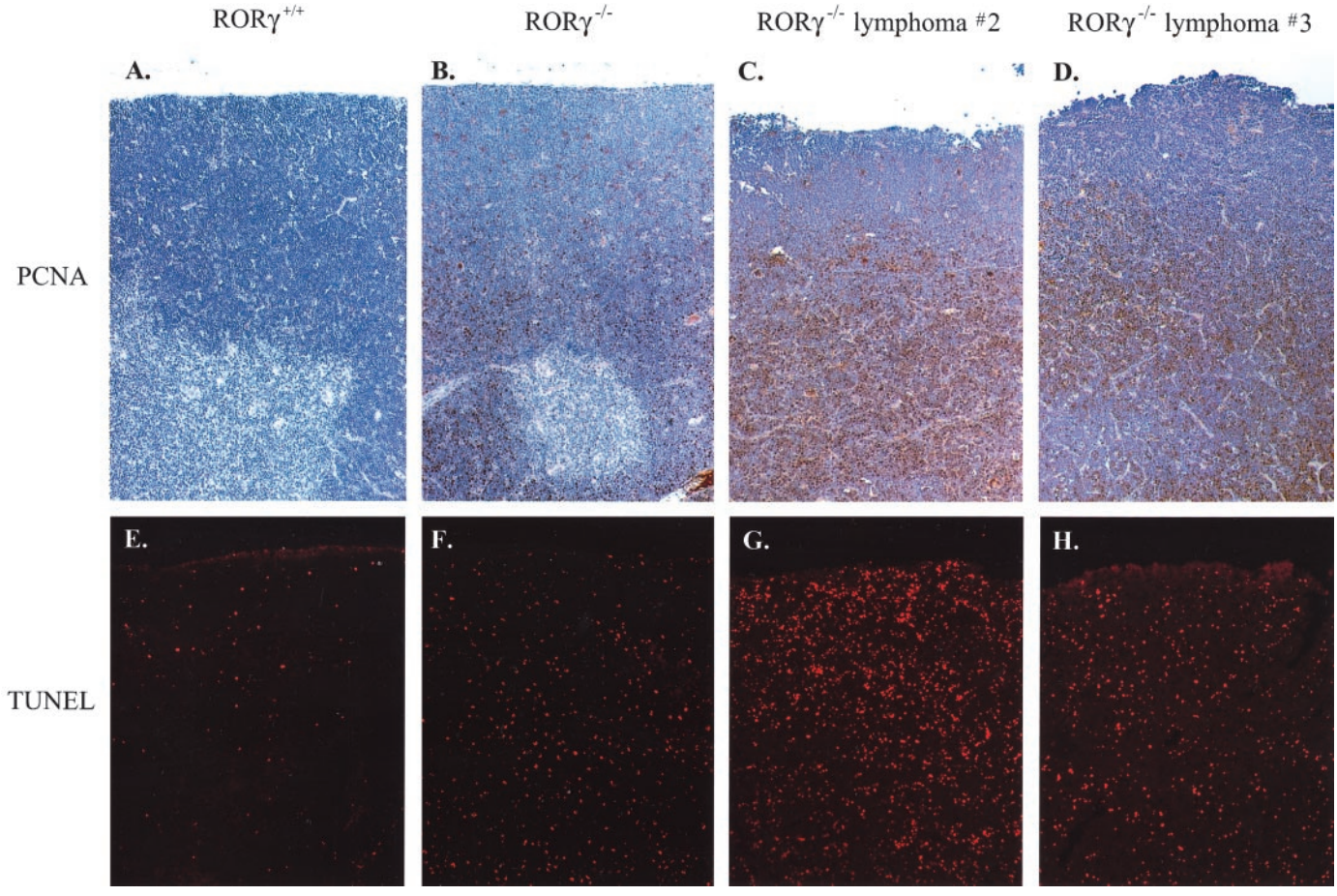

Fig. 3. Both cellular proliferation and apoptosis are increased in RORγ$^{-/-}$ T-cell lymphomas. Sections of wild-type and RORγ$^{-/-}$ thymus and two RORγ$^{-/-}$ T-cell lymphomas (nos. 2 and 3) were examined by TUNEL and PCNA staining. Increased PCNA (*A–D*; ×20) and TUNEL (*E–H*; ×10) staining was observed in thymus from RORγ$^{-/-}$ mice and RORγ$^{-/-}$ T-cell lymphomas. The sections are representative for tissues obtained from several different mice.

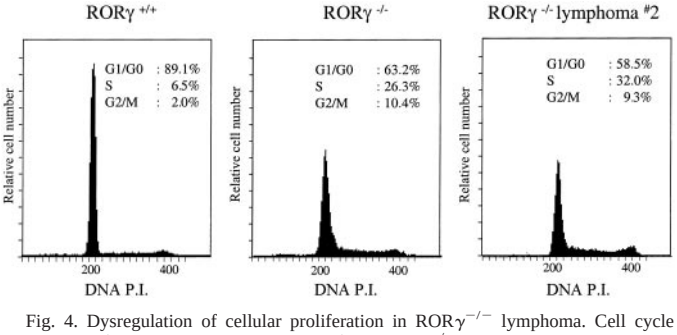

Fig. 4. Dysregulation of cellular proliferation in RORγ$^{-/-}$ lymphoma. Cell cycle distribution of lymphoblastic cells obtained from RORγ$^{-/-}$ lymphomas was determined by flow cytometric analysis as described in "Materials and Methods" and compared with those of wild-type and RORγ$^{-/-}$ thymocytes. Cell cycle analyses of samples obtained from several different mice were averaged and plotted in Table 1.

of cells undergoing apoptosis (Fig. 3, *E–H*). Apoptotic cells were found consistently throughout the tumor and appeared not to be restricted to a certain region. As reported previously, in contrast with thymocytes from wild-type mice, RORγ$^{-/-}$ thymocytes undergo accelerated "spontaneous" apoptosis when placed in culture (25, 26). To determine whether RORγ$^{-/-}$ lymphoblastic cells are resistant to "spontaneous" apoptosis or whether they exhibit a similar sensitivity as RORγ$^{-/-}$ thymocytes, we isolated and cultured lymphoblastic cells from different tumors. As shown in Fig. 5*A*, RORγ$^{-/-}$ lymphoblastic cells placed in culture underwent apoptosis at an accelerated rate very similar to that of RORγ$^{-/-}$ thymocytes, as indicated by the rapid increase in Annexin V binding. In both cases, >90% of the cells underwent apoptosis after 18 h in culture, as compared with only 20% of wild-type thymocytes.

As reported previously, suppression of the expression of the antiapoptotic gene *Bcl-X*$_L$ appears to play an important role in the observed accelerated apoptosis in RORγ$^{-/-}$ thymocytes (25, 26). As in RORγ$^{-/-}$ thymocytes, expression of Bcl-X$_L$ mRNA was barely detectable in RORγ$^{-/-}$ lymphoblastic cells (Fig. 6), suggesting that this repression may also be part of the mechanism causing accelerated "spontaneous" apoptosis in lymphoblastic cells. However, further analysis identified several important differences between RORγ$^{-/-}$ thymocytes and RORγ$^{-/-}$ lymphoblastic cells. In contrast with the accelerated apoptosis in RORγ$^{-/-}$ thymocytes, which is inhibited by the CDK2 inhibitor roscovitine (25, 26), apoptosis in RORγ$^{-/-}$ lymphoblastic cells was resistant to inhibition by 50 μM roscovitine (Fig. 5*B*). In addition, a small number of lymphoblastic cells from several tumors continued to survive and proliferate after prolonged culture. From these cells, we were able to establish several lymphoblast-like cell lines. In contrast, none of the cultured wild-type or RORγ$^{-/-}$ thymocytes were able to survive or continued to proliferate over a prolonged time period.

Table 1 *Cell cycle analysis of RORγ$^{-/-}$ lymphoma cells and thymocytes from RORγ$^{+/+}$ and RORγ$^{-/-}$ mice*

Source of thymocytes	G_1-G_0 (%)	S (%)	G_2-M (%)
RORγ$^{+/+}$ (n = 4)	84.3 ± 2.8	4.7 ± 0.8	10.8 ± 2.3
RORγ$^{-/-}$ (n = 4)	54.2 ± 5.9	25.9 ± 4.5	12.1 ± 3.5
RORγ$^{-/-}$ lymphoma (n = 3)	58.8 ± 0.3	32.1 ± 0.4	9.0 ± 0.2

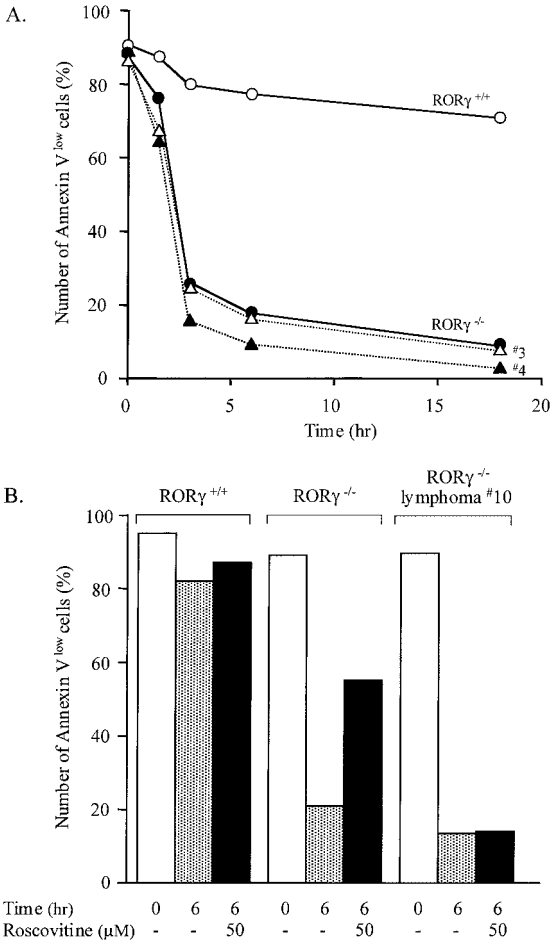

Fig. 5. Accelerated "spontaneous" apoptosis in RORγ$^{-/-}$ lymphoblastic cells. *A*, freshly isolated RORγ$^{-/-}$ lymphoma cells were cultured, and at different time intervals, cells were analyzed for Annexin V binding by flow cytometry as described in "Materials and Methods." The rate of apoptosis was compared with that of wild-type and RORγ$^{-/-}$ thymocytes. The percentage of cells with low Annexin V binding (cells not undergoing apoptosis) was calculated and plotted. *B*, accelerated "spontaneous" apoptosis in RORγ$^{-/-}$ lymphoblastic cells is resistant to the CDK2-inhibitor roscovitine (50 μM). Cells were cultured in the presence or absence of roscovitine and 6 h later analyzed for Annexin V binding. In contrast to wild-type and RORγ$^{-/-}$ thymocytes, "spontaneous" apoptosis in RORγ$^{-/-}$ lymphoblastic cells was not inhibited by roscovitine. The results shown are representative for three independent analyses.

Previous studies have linked alterations in the expression of several genes to T-cell lymphoma development in the thymus and include loss of tumor suppressor gene expression and gain in oncogene expression (28–32). For example, mice deficient in the expression of p53 have been reported to be highly susceptible to the development of thymic lymphomas (32, 33), and ectopic expression of c-*myc* has been shown to promote development of T-cell lymphomas (34). To determine whether some of these genes might be involved in the development of T-cell lymphomas in RORγ$^{-/-}$ mice, we compared their expression in lymphoblastic cells from different RORγ$^{-/-}$ lymphomas, with those of thymocytes from 8-week-old wild-type mice and RORγ$^{-/-}$ mice that did not yet exhibit an enlarged thymus. As shown in Fig. 6, expression of p53 mRNA in thymocytes from RORγ$^{-/-}$ mice and lymphomas was comparable with those from wild-type mice. Similarly, no change in the expression of *Brca2* (Fig. 6) or c-*myc* (not shown) mRNA was found between RORγ$^{-/-}$ thymocytes, normal thymocytes, and lymphoma cells. These results do not indicate a link between the expression of *Brca2*, *p53*, or c-*myc* and the formation of T-cell lymphomas in RORγ$^{-/-}$ mice. Examination of RORγ expression in several T-cell lymphomas derived from p53$^{-/-}$ mice showed little change in RORγ expression, indicating that loss of p53 does not affect the expression of RORγ.

T-lymphocyte maturation in the thymus is a well-defined, multistep process that involves proliferation, differentiation, apoptosis, selection, and commitment to different lineages. Early during thymopoiesis, the immature CD4$^-$CD8$^-$CD44$^+$CD25$^-$ progenitors, which represent a minority (3–5%) in the adult thymus, differentiate via two intermediate stages into CD4$^-$CD8$^-$CD44$^-$CD25$^-$ pre-T cells. These cells then differentiate further into CD4$^+$CD8$^+$ double-positive thymocytes, which constitute the majority (80–85%) of the thymocyte population. The majority of double-positive cells undergo apoptosis (death by neglect or by negative selection), and only a small fraction of the surviving, positively selected, double-positive cells mature further into single-positive CD4$^+$ helper and single-positive CD8$^+$ cytotoxic lineages. To assess their immunophenotype, lymphoblastic cells isolated from various RORγ$^{-/-}$ lymphomas were examined by flow cytometry for the expression of CD4, CD8, CD44, and CD25 antigens. Previously, we reported that the thymus of RORγ$^{-/-}$ mice contained a higher percentage of the double-negative subpopulation compared with wild-type mice (25, 26), whereas their double-positive population was decreased. In addition, the double-positive population shifted and exhibited reduced levels of CD4. As shown in Fig. 7*A* and Table 2, the percentages of the different CD4/CD8 subpopulations varied substantially among lymphomas and were different from those of wild-type and RORγ$^{-/-}$ mice. Although in most tumors the percentage of double-positives decreased and the number double-negative and single-positive CD8$^+$ increased, the tumors could be divided into three different groups based on their CD4 and CD8 expression. Some RORγ$^{-/-}$ lymphomas (*e.g.*, lymphoma 1 and 6; Table 2) still contained a high percentage of double-positive cells, whereas others contained a high percentage of double-negative

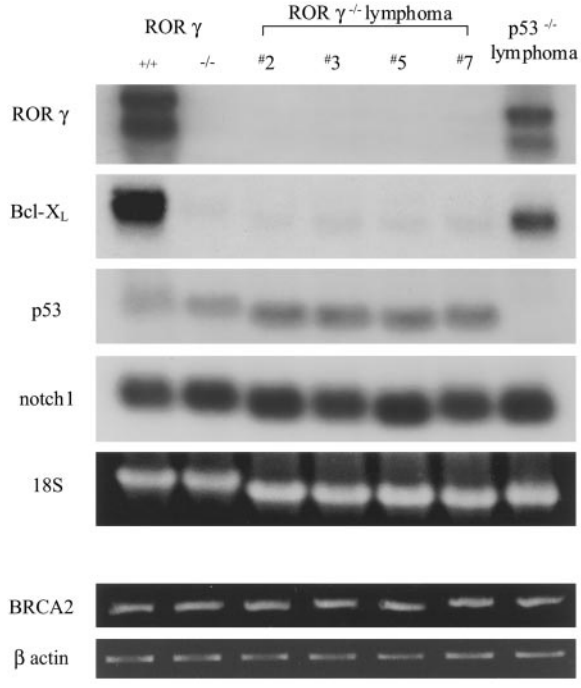

Fig. 6. Comparison of the expression of *RORγ*, *Bcl-X*$_L$, *p53*, and *Brca2* in thymocytes from wild-type and RORγ$^{-/-}$ mice and various T-cell lymphomas. Total RNA was isolated from wild-type and RORγ$^{-/-}$ thymocytes and lymphoblastic cells from T-cell lymphomas of RORγ$^{-/-}$ and p53$^{-/-}$ mice and examined by Northern blot analysis using radiolabeled probes for *RORγ*, *Bcl-X*$_L$, *p53*, and *notch-1*. Expression of *Brca2* mRNA was examined by RT-PCR as described in "Materials and Methods." *RORγ* and *p53* expression was lost in cells from RORγ$^{-/-}$ and p53$^{-/-}$ mice, respectively. *Bcl-X*$_L$ expression was suppressed in cells from RORγ$^{-/-}$ mice but was still highly expressed in p53$^{-/-}$ T-cell lymphoma cells. No changes in *notch-1* expression were observed.

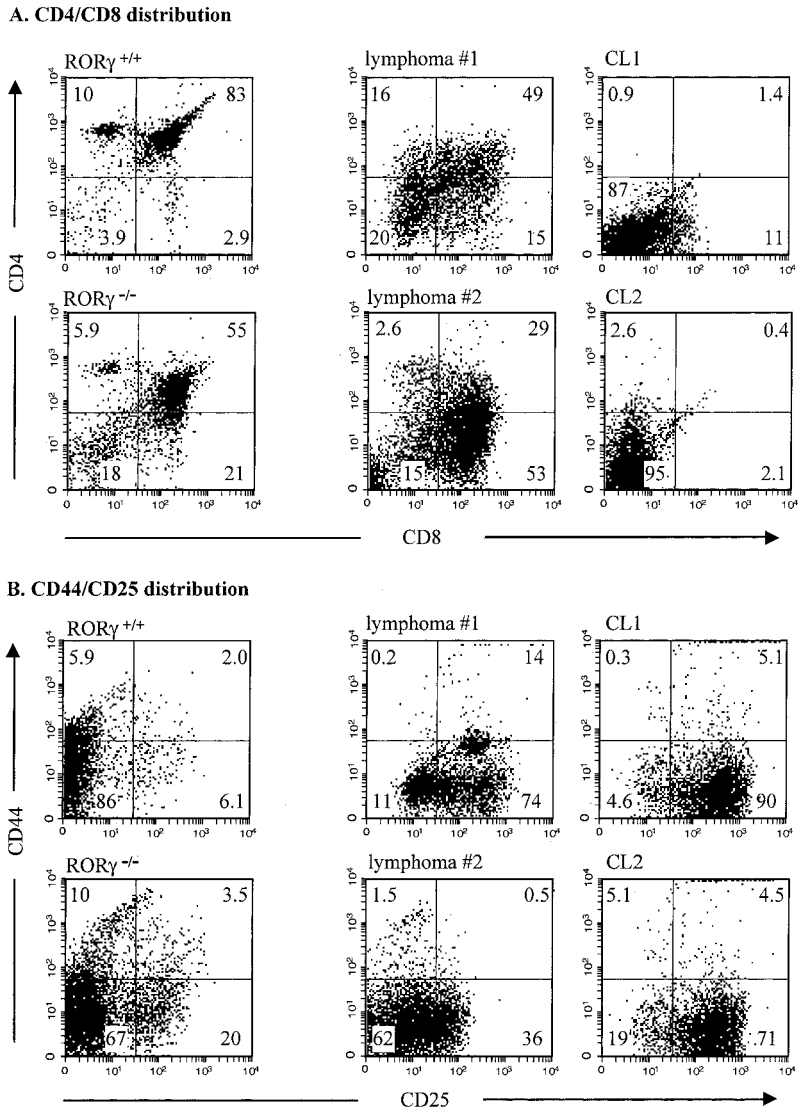

Fig. 7. Aberrant T-cell development in RORγ$^{-/-}$ mice. *A*, wild-type and RORγ$^{-/-}$ thymocytes and lymphoblastic cells from RORγ$^{-/-}$ lymphoma nos. 1 and 2 were analyzed for the presence of CD4 and CD8 (*A*) or CD44 and CD25 antigen (*B*) by flow cytometry as described in "Materials and Methods." CD4, CD8, CD44, and CD25 were also examined in the lymphoblastic cell lines CL1 and CL2 that were established from RORγ$^{-/-}$ lymphoma nos. 1 and 2, respectively.

CD4^{-}CD8^{-} (*e.g.*, lymphomas 5, 9, and 10) or single-positive CD8^{+} (*e.g.*, lymphomas 4, 7, and 8) cells. Analysis of CD4 and CD8 expression in blood lymphocytes from mice with lymphomas showed consistently the presence of a high percentage of double-positive cells, which normally are absent, and a decrease in the percentage of single-positive CD4^{+} cells. Examination of CD25 and CD44 cell surface antigens showed that the percentage of the CD44^{-}CD25^{+} thymocyte subpopulation in RORγ$^{-/-}$ mice was increased compared with wild-type mice (Fig. 3*B*). However, in RORγ$^{-/-}$ lymphomas, the majority of cells were CD44^{-}CD25^{-}, but the population of CD44^{-}CD25^{+} cells was also dramatically enhanced. Initially the cell lines established from various tumors exhibited consistently a similar and homogeneous phenotype. After 1 month of culture, lymphoblastic cells were predominantly CD4^{-}CD8^{-} and either CD44^{-}CD25^{+} or CD44^{-}CD25^{-}. These results indicate that the pattern of CD4, CD8, and CD25 expression changed upon continuous culture. The large differences seen in CD4/CD8 subpopulations in various tumors may reflect a similar process of selection and progression.

To determine the immunophenotype of the actively proliferating thymocytes, cells in the S-phase were selected and analyzed by flow cytometry for CD4 and CD8 expression. In contrast with normal CD4^{+}CD8^{+} thymocytes, which do not proliferate, certain tumors contained a relatively high number of cycling CD4^{+}CD8^{+} and/or CD4^{-}CD8^{+} lymphoblastic cells (not shown). These results provide further evidence for the hypothesis that lack of RORγ expression leads to dysregulation of proliferation and differentiation.

As mentioned above, a few homozygous mice of each genetic background survived after 1 year. Necropsy of these mice showed the presence of a smaller, normal thymus. Examination of their CD4CD8 subpopulations showed a pattern identical to that observed in thymocytes from 6–8-week-old, tumor-free RORγ$^{-/-}$ mice (not shown). The percentage of double-negative cells was increased, and that of double-positive cells decreased. The number of S-phase cells was enhanced (~22%) compared with 5% in 1-year-old control mice but was not as high (~26%) as in RORγ$^{-/-}$ thymocytes from 6–8-week-old mice.

DISCUSSION

In this study, we demonstrate that mice lacking both normal RORγ alleles are highly susceptible to development of T-cell lymphomas. The onset of tumor formation occurs during early stages of life; at 122 days of age, 50% of the RORγ$^{-/-}$ mice of mixed genetic background had become moribund or died. No significant difference in median survival was observed between RORγ$^{+/-}$ *versus* RORγ$^{+/+}$ mice of mixed genetic background, suggesting that there is no gene dosage

Table 2 *Immunophenotype of RORγ$^{-/-}$ thymic lymphomas and lymphoma-derived cell lines*

Lymphoblastic cells isolated from thymic lymphomas (nos. 1–11) were analyzed for their expression of CD4 and CD8 by flow cytometry. The percentages of the different CD4/CD8 subpopulations in RORγ$^{-/-}$ lymphomas were calculated and compared with those of thymocytes isolated from RORγ$^{+/+}$ and RORγ$^{-/-}$ mice and p53$^{-/-}$ lymphomas (nos. 12 and 13). The percentages for RORγ$^{+/+}$ and RORγ$^{-/-}$ thymocytes were derived from a previous study (25). CL1, CL2, and CL3 represent RORγ$^{-/-}$ lymphoma-derived cell lines.

	Thymocytes			
Source of thymocytes	DN[a]	DP	SP(CD4)	SP(CD8)
RORγ$^{+/+}$ (n = 3)	2.6	86.4	6.6	2.7
RORγ$^{-/-}$ (n = 3)	10.9	78.5	3.3	3.8
RORγ$^{-/-}$ lymphoma				
1	20	49	15	16
2	15	29	2.6	53
3	15	28	0.5	57
4	3.9	8.0	0.1	88
5	63	5.6	9.4	22
6	19	52	0.9	28
7	14	24	1.8	61
8	37	4.6	0.6	58
9	57	16	15	12
10	55	27	3.5	14
11	19	40	0.6	40
p53$^{-/-}$ lymphoma				
12	5.8	93	1.0	0.03
13	8.2	47	0.4	43
Lymphoma cell lines				
CL1	87	1.2	1.3	10
CL2	93	1.1	4.9	0.4
CL5	57	4.6	4.5	34

[a] DN, double negative; DP, double positive; SP, single positive.

effect on survival as a result of the loss of one or two alleles of RORγ. All but 1 of 17 moribund RORγ$^{-/-}$ mice examined revealed the presence of a thymic lymphoma, and no other tumor types were observed in other organs. Over a period of 1 year, none of the wild-type or heterozygous mice developed a thymic lymphoma. RORγ$^{-/-}$ mice of a 129/SvEv × B6D2 × C57BL/6 mixed background and RORγ$^{-/-}$ 129/SvEv mice developed lymphomas at very similar rates, and in both genetic backgrounds, a few mice were able to survive longer than 1 year. These mice did not have an enlarged thymus or any other tumor, and the characteristics of the thymocytes were very similar to those described for 8-week-old, tumor-free RORγ$^{-/-}$ mice. Susceptibility to the development of thymic lymphomas has been reported to vary greatly between mouse strains (35). Little difference in susceptibility to T-cell development was observed between 129/SvEv × B6D2 × C57BL/6 mixed background and RORγ$^{-/-}$ 129/SvEv mice. We are backcrossing the RORγ$^{-/-}$ phenotype into other strains to examine this further.

The high incidence of lymphoma in RORγ$^{-/-}$ mice may be a result of dysfunction of important physiological events during thymocyte maturation. Previous studies identified changes in thymocyte proliferative activity, rate of apoptosis, and shifts in CD4/CD8 subpopulations in thymi from 8-week-old RORγ$^{-/-}$ mice in which the thymus was not yet enlarged. The development of lymphomas in RORγ$^{-/-}$ mice is associated with a further increase in proliferative activity, as indicated by increased PCNA staining and a 5-fold increase in the number of S-phase cells in lymphomas compared with thymi from wild-type mice. Analysis of the immunophenotype of S-phase cells indicated that the population of actively dividing cells consisted largely of double-negative and single-positive CD8$^+$ cells. These observations further support the hypothesis that the lack of RORγ expression disturbs homeostasis by altering proliferation and maturation of thymocytes.

Tumor formation is a multistep process that usually requires multiple changes in gene expression. Thus, the formation of lymphomas in RORγ$^{-/-}$ mice likely involves changes in the expression of additional genes. Such changes may arise from an enhanced probability of individual cells to acquire genetic alterations in genes that make them escape negative growth control mechanisms and normal differentiation programs, which as a consequence lead to increased susceptibility to the development of T-cell lymphoma. In other *in vivo* lymphoma models, failure to repair DNA breaks properly during the process of DNA rearrangements has been thought to lead to increased frequency of chromosomal translocations and inversion. The latter is thought to result in abrogation of the expression of tumor suppressor genes and/or activation of proto-oncogenes.

Loss of expression of the tumor suppressors *p53* or *Brca2*, or overexpression of the proto-oncogene c-*myc*, have been reported to promote thymic lymphoma development in mice (31, 36, 37, 38). These genes could potentially be playing a role in lymphoma formation in RORγ$^{-/-}$ mice. However, comparison of the expression of *p53*, *Brca2*, and c-*myc* mRNA in thymocytes from wild-type and RORγ$^{-/-}$ mice, and RORγ$^{-/-}$ lymphoblastic cells showed very similar levels of expression of these genes. These results suggest that these tumor suppressor and proto-oncogenes are unlikely to be involved in lymphoma formation in RORγ$^{-/-}$ mice. Our results also demonstrated that RORγ expression was not affected in p53$^{-/-}$ mice, suggesting that RORγ expression is not regulated by p53. The conclusion that the induction of thymic lymphomas in p53 or RORγ-deficient mice involves different mechanisms is supported by several other differences in phenotype between these knockout mice. The immunophenotype of the lymphomas in the two knockout strains are quite different. p53$^{-/-}$ mice primarily yield lymphomas with a double-positive phenotype, whereas single-positive CD8$^+$ and double-negative cells become abundant in RORγ$^{-/-}$ lymphomas. Mice deficient in p53 exhibit a high rate of lymphoma formation, whereas p53 heterozygous mice also formed lymphomas but at a reduced rate in contrast with RORγ$^{+/-}$ mice, which do not form lymphomas over a 1-year period. These results indicate that the mechanisms by which RORγ and p53 deficiency lead to lymphoma formation are different.

As reported previously, RORγ$^{-/-}$ thymocytes undergo accelerated "spontaneous" apoptosis when placed in culture (25, 26). This induction appears to be related in part to the lack of expression of the antiapoptotic gene *Bcl-X$_L$*; the expression of several other Bcl family members was not greatly altered in RORγ$^{-/-}$ thymocytes (25). The accelerated apoptosis appears not to involve Fas and Fas ligand (25, 26). The critical role of Bcl-X$_L$ suppression in accelerated apoptosis is supported by findings showing that ectopic expression of Bcl-X$_L$ inhibits this apoptosis (26). These results indicate that RORγ regulates directly or indirectly the expression of Bcl-X$_L$; however, the precise mechanism by which RORγ controls Bcl-X$_L$ has yet to be elucidated. In addition to the repression of Bcl-X$_L$, RORγ$^{-/-}$ thymocytes express high levels of CDK2 activity that appears to be negatively controlled by Bcl-X$_L$ (26). The increase in CDK2 activity plays an important role in increased apoptosis, because the CDK2 inhibitor roscovitine inhibits spontaneous apoptosis in RORγ$^{-/-}$ thymocytes. In this study, we demonstrate that the transition of RORγ$^{-/-}$ thymocytes to RORγ$^{-/-}$ lymphoblastic cells is not accompanied by a loss in the ability to undergo apoptosis because tumor sections stained for TUNEL showed a high number of apoptotic cells. In fact, tumor cells *in vitro* undergo accelerated "spontaneous" apoptosis at a rate comparable with that of RORγ$^{-/-}$ thymocytes and wild-type thymocytes and, therefore, did not become resistant to apoptosis. In contrast with RORγ$^{-/-}$ thymocytes, apoptosis in RORγ$^{-/-}$ lymphoblastic cells was not inhibited by the CDK2 inhibitor roscovitine, indicating that CDK2 activity does not appear to play a role in the mechanism by which RORγ$^{-/-}$ lymphoblastic cells undergo apoptosis. Although increased apoptosis and lymphoma formation appear at first sight contradictory, increased

proliferation has been found associated with increased apoptosis in several hyperplastic diseases. For example, increased proliferation and apoptosis have been observed in several tumor models, including tumor formation in $p53^{-/-}CK^{+}$ mice, which are deficient in p53 and exhibit increased expression of CK2a (39), and in E2- and E47-deficient mice (28). Transgenic mice, in which expression of the helix-loop-helix gene *Id1* is specifically targeted to T cells, also develop T-cell lymphomas while thymocytes undergo massive cell death (30).

Early T-cell development at the double-negative stage can be characterized by the expression of CD44 and CD25 cell surface markers. Multipotent progenitor cells marked as $CD44^{+}CD25^{-}$ become committed to the T-cell lineage and differentiate through the $CD44^{+}CD25^{+}$, $CD44^{-}CD25^{+}$, $CD44^{-}CD25^{-}$ and then into the double-positive $CD4^{+}CD8^{+}$ stages (40, 41). The molecular mechanisms that regulate the transition between these stages are not yet precisely understood. Expression of RORγ2 is restricted to double-positive cells, where it is most highly expressed, and to very immature double-negative $CD44^{+}CD25^{-}$ cells (22). Thus, RORγ expression is induced when double-negative cells differentiate into double-positive cells and is subsequently down-regulated during the differentiation of double-positive into single-positive cells. RORγ may act both as a repressor and as an activator of transcription and as such regulate positively or negatively several steps in the maturation of double-negative to single-positive cells. The double-negative to double-positive transition corresponds to a critical checkpoint that depends on the productive rearrangement and expression of TCRβ, which associates with the CD3 complex at the cell surface of late double-negative thymocytes to form the pre-TCR. Signals through the pre-TCR lead to proliferation and differentiation into double-positive, and evidence has been provided indicating that this appears also to result in an up-regulation of RORγ2 expression (21). It has been suggested that RORγ2 may influence VαJα rearrangements. It is clear that thymocyte precursor cells in $ROR\gamma^{-/-}$ mice can differentiate into double-positive and fully mature single-positive $CD4^{+}$ and single-positive $CD8^{+}$ cells, and that lack of RORγ expression does not cause a block at a specific stage of differentiation (25, 26). The high lymphoma frequency may be a result of dysregulation of specific functions, such as proliferation and apoptosis, at one or more critical steps in the differentiation of double-negative to double-positive thymocytes. The observed increase in the number of cycling cells and the accelerated apoptosis appear to support this hypothesis.

In summary, our results show that mice deficient in RORγ expression are highly susceptible to the development of T-cell lymphoma. This increase in susceptibility may relate to alterations in homeostasis attributable to changes in apoptosis, proliferation, and differentiation. Increased proliferation may result in an enhanced probability to acquire genetic alterations that lead to additional changes in the expression of tumor suppressor and proto-oncogenes and which as a consequence lead to tumor formation. Our study also opens the possibility of a role for RORγ in human cancer. Future identification of RORγ target genes and changes in gene expression in $ROR\gamma^{-/-}$ thymocytes will help to elucidate the regulatory role of RORγ in normal thymopoiesis and in the development of T-cell lymphoma.

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