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ROR1 can interact with TCL1 and enhance leukemogenesis in Eµ-TCL1 transgenic mice

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Receptor tyrosine kinase-like orphan receptor 1 (ROR1) is an oncoembryonic antigen found on chronic lymphocytic leukemia (CLL) B cells, but not on normal adult tissues. We generated transgenic (Tg) mice with human ROR1 regulated by the murine Ig promoter/enhancer. In contrast to nontransgenic littermates, such animals had B-cell–restricted expression of ROR1 and could develop clonal expansions of ROR1^{bright}CD5^{+}B220^{low} B cells resembling human CLL at ≥15 mo of age. Because immune-precipitation and mass spectrometry studies revealed that ROR1 could complex with T-cell leukemia 1 (TCL1) in CLL, we crossed these animals with Eµ-TCL1-Tg (TCL1) mice. Progeny with both transgenes (ROR1 × TCL1) developed CD5^{+}B220^{low} B-cell lymphocytosis and leukemia at a significantly younger median age than did littermates with either transgene alone. ROR1 × TCL1 leukemia B cells had higher levels of phospho-AKT than TCL1 leukemia cells and expressed high levels of human ROR1, which we also found complexed with TCL1. Transcriptome analyses revealed that ROR1 × TCL1 leukemia cells had higher expression of subnetworks implicated in embryonic and tumor-cell proliferation, but lower expression of subnetworks involved in cell–cell adhesion or cell death than did TCL1 leukemia cells. ROR1 × TCL1 leukemia cells also had higher proportions of K_i-67–positive cells, lower proportions of cells undergoing spontaneous apoptosis, and produced more aggressive disease upon adoptive transfer than TCL1 leukemia cells. However, treatment with an anti-ROR1 mAb resulted in ROR1 down-modulation, reduced phospho-AKT, and impaired engraftment of ROR1 × TCL1 leukemia cells. Our data demonstrate that ROR1 accelerates development/progression of leukemia and may be targeted for therapy of patients with CLL.

mouse model | monoclonal antibody | AKT | immune therapy

Receptor tyrosine kinase-like orphan receptor 1 (ROR1) is a type 1 tyrosine kinase surface protein expressed on chronic lymphocytic leukemia (CLL) B cells, but not on nonmalignant postpartum tissues (1). Expression of ROR1 ordinarily is confined to early embryogenesis, where it contributes to organogenesis (2–4). However, ROR1 is not detected on postpartum tissues, including hematopoietic stem cells, except on a small subset of B-cell precursors, called hematogones (5). We and others have found ROR1 expressed on the neoplastic cells of patients with CLL (1, 6, 7), other B-cell lymphomas (8), acute leukemias (5, 9), and on any one of a variety of solid tumors (10–13). However, it still is uncertain whether ROR1 plays a role in cancer development and/or disease progression.

ROR1 has an extracellular domain that is essential for ligand binding and potentially for intracellular signaling (14). Although it has a putative kinase domain, ROR1 might function as a pseudokinase, serving instead as a cofactor for other signaling proteins (13). We found that ROR1 could serve as a receptor for Wnt5a, which could provide a survival/growth signal to CLL cells from the microenvironment (1). Also, ROR1 could enhance the survival and proliferation of ROR1-negative CLL cells (e.g., MEC-1 cell line) (11). On the other hand, silencing ROR1 with shRNA could induce CLL cells to undergo apoptosis (15).

The T-cell leukemia 1 (TCL1) oncogene similarly is overexpressed in CLL, where it may contribute to leukemogenesis (16, 17). Eµ-TCL1 transgenic (TCL1 Tg) mice, which express human TCL1 under the control of a B-cell–specific promoter, develop a CD5^{+} B-cell leukemia with features of human CLL (18, 19). Enlarged lymph nodes, splenomegaly, and elevated blood lymphocyte counts are noted with disease progression. Numerous studies indicate that the TCL1 Tg mouse model of CLL may be a useful tool for defining the relevance of genes thought to contribute to pathogenesis in CLL, such as TNFSF13B, HS1, TIR8, ID4, or miR15A (20–26).

To investigate the functional significance of ROR1 in the development and/or progression of CLL, we generated C57BL/6 mice transgenic for human ROR1 under the control of the murine Ig promoter/enhancer, which drives B-cell–restricted expression of ROR1. We also crossed ROR1 Tg mice with TCL1 Tg mice and examined the effect of expression of ROR1 on the development and progression of leukemia in the ROR1 × TCL1 animals compared with that observed in TCL1 Tg mice.

Results

ROR1 Transgenic Mice. We generated transgenic mice with the human ROR1 cDNA under the control of the mouse IgH promoter/enhancer, providing for B-cell–restricted expression of ROR1

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(Fig. S1*A*). *ROR1* transgenic (ROR1 Tg) mice developed mature B cells in the blood, spleen, marrow, and peritoneal cavity that constitutively expressed ROR1, as assessed by flow cytometry (Fig. 1 *A–D*). ROR1 Tg mice also developed increased numbers of splenic lymphocytes relative to that of littermates lacking the *ROR1* transgene (Fig. S1*B*). Very late in life (≥15 mo), some (3 of 65, 5%) of the ROR1 Tg mice developed lymphocytosis and splenomegaly due to accumulations of clonal ROR1^{bright}CD5$^+$B220^{low} B cells that resembled human CLL (Fig. 1*E* and Fig. S2*A*) and that was not observed in age-matched littermates. Such cells expressed higher levels of ROR1 than the nonleukemic CD5$^-$B220^{hi} B cells of ROR1 Tg mice (Fig. 1*E*).

Interaction of ROR1 with TCL1. TCL1 Tg mice that have the human TCL1 under the same B-cell–specific promoter also develop a CLL-like disease, but at around 7–9 mo of age. These animals generally succumb to this disease between 13 and 18 mo of age with massive splenomegaly and lymphocycytosis (18). We examined the splenic leukemia cells that developed in TCL1 mice and found that they do not express mouse ROR1 (Fig. 1*F*), indicating that ROR1 is not necessary for leukemogenesis in this model.

However, the leukemia cells of patients with CLL almost invariably coexpress both ROR1 (1, 6, 7) and TCL1 (16, 17). Furthermore, mass spectrometry analyses repeatedly detected TCL1 in immune precipitates of ROR1, but not in immune precipitates of control Ig (Fig. S3*A*). This association was confirmed by immunoblot analyses of anti-TCL1 immune precipitates, which we found contained ROR1 (Fig. S3*B*).
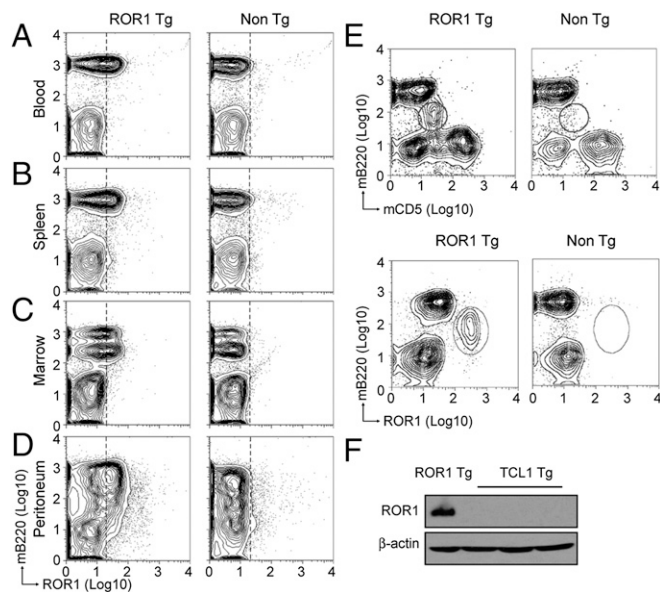
Fig. 1. Generation and characterization of human ROR1 Tg mice. Contour plots depicting the fluorescence of lymphocytes isolated from blood (*A*), spleen (*B*), marrow (*C*), or peritoneum (*D*) of representative 4-mo-old ROR1 Tg mice (*Left* column) or control littermates (*Right* column) after staining the cells with fluorochrome-conjugated mAb specific for B220 (*y* axis) and human ROR1 (*x* axis). The vertical dotted line depicts the fluorescence threshold for which the cells to the *Right* are considered positive for ROR1. (*E*) Contour plots depicting the fluorescence of lymphocytes isolated from blood of a representative 18-mo-old ROR1 Tg mouse (*Left*) or control littermate (*Right*) after staining the cells with fluorochrome-conjugated mAb specific for B220 and CD5 (*Upper*) or human ROR1 (*Lower*), as indicated in the labels. (*F*) Immunoblot analyses of cell lysates prepared from splenic lymphocytes from a ROR1 Tg mouse (*Far Left* lane) or the CD5$^+$B220^{low} splenic leukemia B cells from each of three unrelated TCL1 Tg mice, and then probed with a mAb that binds either human or mouse ROR1 or β-actin.

To interrogate the influence of ROR1 in the TCL1 mouse model, we crossed ROR1 Tg mice with TCL1 Tg mice and found that ROR1 × TCL1 Tg mice typically had higher percentages of CD5$^+$B220^{low} B cells among their blood lymphocytes than did littermate controls having only the TCL1 transgene (Fig. 2*A*). Groups of ROR1 × TCL1 and TCL1 Tg mice were monitored biweekly for development of CD5$^+$B220^{low} B cells in the blood via flow cytometry. At 5 mo of age, the median proportion of blood lymphocytes that were CD5$^+$B220^{low} B cells was 6.8% (mean = 10.4 ± 1.9, *n* = 30) in ROR1 × TCL1 Tg mice, whereas it was 3.3% (mean = 5.4 ± 1.3, *n* = 30, *P* = 0.018) in littermates that had only TCL1 (Fig. 2*B*). Similarly, at 6 mo of age the median proportion of CD5$^+$B220^{low} B cells was 10.9% (mean = 17.4 ± 2.5, *n* = 30) in ROR1 × TCL1 Tg mice, but only 8.4% (mean = 10.9 ± 1.7, *n* = 30) in TCL1 Tg mice (*P* = 0.017). Analysis of these data using a linear mixed effect model indicated that ROR1 significantly accelerated expansion of CD5$^+$B220^{low} B cells in TCL1 Tg mice (*P* = 0.033). Such expansions of CD5$^+$B220^{low} B cells led to development of clonal leukemia in each animal (Fig. S2), resulting in lymphocytosis and splenomegaly resembling human CLL, as assessed on necropsy (Fig. S4). The earlier development of CD5$^+$B220^{low} B-cell leukemia in ROR1 × TCL1 mice was associated with a significantly shorter median survival (survival of 50.6 wk, *n* = 26) than that observed for TCL1 Tg mice (57.7 wk, *n* = 26, *P* = 0.009) (Fig. 2*C*), with death being secondary to lymphoproliferative disease.

As in human CLL, we noted that treatment of whole-cell lysates with anti-TCL1 immune-precipitated ROR1, which was not detected in anti-TCL1 immune precipitates of whole-cell lysates of TCL1 leukemia cells (Fig. 2*D*). Although TCL1 also can be detected in nuclear extracts, the interaction between ROR1 and TCL1 most likely occurs in proximity to the plasma membrane, as ROR1 was detected only in lysates prepared from the cytosol and plasma membrane, but not from lysates prepared from isolated nuclei (Fig. S3*C*).

We performed microarray transcriptome analyses on isolated CD5$^+$B220^{low} splenic leukemia cells that developed in ROR1 × TCL1 Tg mice (*n* = 4) or TCL1 Tg mice (*n* = 4). This revealed that the ROR1 × TCL1 leukemia cells shared common gene-expression signatures that were distinct from those of TCL1 leukemia cells (Fig. 3*A*). Subnetwork analyses revealed 51 subnetworks that were expressed at different levels between the two types of leukemia, 21 of which had Z scores in excess of 0.8 and a false discovery rate (FDR) of less than 0.05 (Fig. 3*A*). Activity Z scores for each of these 51 subnetworks and their associated functional annotation are presented in Fig. S5*A*. ROR1 × TCL1 leukemia cells had higher-level expression of subnetworks associated with protein-kinase activation and proliferation of embryonic or tumor cells, but had lower-level expression of subnetworks associated with cell–cell adhesion or induction of apoptosis, than did TCL1 leukemia cells (Fig. S5 *A* and *B*). We observed that the gene encoding Akt1, a kinase that can be complexed and coactivated by TCL1 (27–31), contributed to many of these subnetworks (Fig. 3*B*). Furthermore, gene-set enrichment analysis (GSEA) of genes encoding proteins involved in targeted signaling pathways in the BIOCARTA database revealed that the leukemias that originated in ROR1 × TCL1 mice had higher expression levels of genes encoding proteins in the AKT pathway compared with the leukemias that originated in TCL1 mice (familywise *P* < 0.01, Table S1). Moreover, the expression levels of 11 of 18 genes in this pathway were moderately, yet consistently, increased in the leukemia cells of ROR1 × TCL1 Tg mice relative to those of TCL1 mice (Fig. S5*C* and Table S2).

We also observed that ROR1 × TCL1 splenic leukemia cells also have higher relative levels of pAKT than splenic TCL1 leukemia cells by immunoblot analysis (Fig. 4*A*). That such increased activation of AKT was associated with enhanced growth and/or survival of ROR1 × TCL1 leukemia cells relative to TCL1 leukemia cells was supported by immunohistochemical and flow cytometric studies of ROR1 × TCL1 or TCL1 leukemia

Fig. 2. ROR1 interacts with TCL1 and accelerates CD5+ B-cell leukemogenesis in TCL1 Tg mice. ROR1 Tg mice were crossed with TCL1 Tg mice and ROR1 × TCL1 and TCL1 Tg age-matched littermates were bled biweekly. The isolated blood mononuclear cells were examined for expression of CD5 and B220 on B cells in the blood. (*A*) Flow cytometric analysis of representative 7-mo-old ROR1 × TCL1 (*Left*), TCL1 (*Center*), or ROR1 (*Right*) Tg mice depicting the expansion of CD5+B220^low B cells in the blood. Contour plots depict staining of lymphocytes, as determined by light scatter characteristics, with fluorochrome-conjugated mAbs specific for mouse B220 (*y* axis) and either CD5 (*Upper*) or ROR1 (*Lower*) on the abscissa. (*B*) Plot depicting the percent of CD5+B220^low lymphocytes in the blood of ROR1 × TCL1 (*Left*) or TCL1 mice (*Right*) at 5, 6, or 7 mo of age, indicating that the expression of ROR1 is associated with a more rapid expansion of CD5+ B220^low B cells in ROR1 × TCL1 Tg mice than in age-matched TCL1 littermates at each time point examined. Welch's *t* test based on the average for each of the three measurements is indicated above when comparing the percent numbers of CD5+ B220^low B cells from ROR1 × TCL1 or TCL1 mice at each age (*n* = 30). (*C*) Survival (Kaplan–Meier) plots

of cohorts of 26 mice each of ROR1 × TCL1 and TCL1 Tg mice. Median survival of ROR1 × TCL1 mice was 50.6 wk and 57.7 wk for TCL1 Tg mice (*P* = 0.009, log rank test). (*D*) Representative lysates prepared from leukemia cells of each of two ROR1 × TCL1 mice (*Left*) or one TCL1 mouse (*Right*) were immunoprecipitated with control IgG or anti-TCL1 mAb that then were examined by immunoblot analysis using antibodies specific for ROR1 (*Upper* row) or TCL1 (*Lower* row), as indicated in the margins.
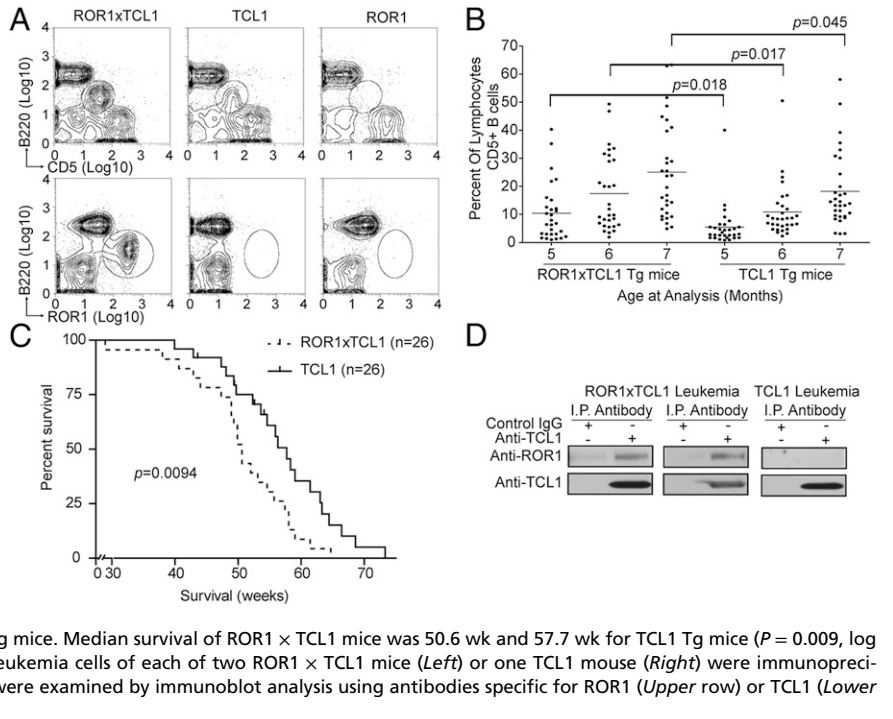
cells engrafted into syngeneic, nonconditioned ROR1 Tg mice. We observed that significantly higher proportions of splenic CD5+B220^low ROR1 × TCL1 leukemia cells stained with the proliferation marker K_i-67 (15.6% ± 0.7%, median = 15.5%, *n* = 3) than did CD5+B220^low TCL1 leukemia cells (9.4% ± 1.5%, median = 9.5%, *n* = 3, *P* = 0.02) (Fig. 4*B*). Furthermore, the
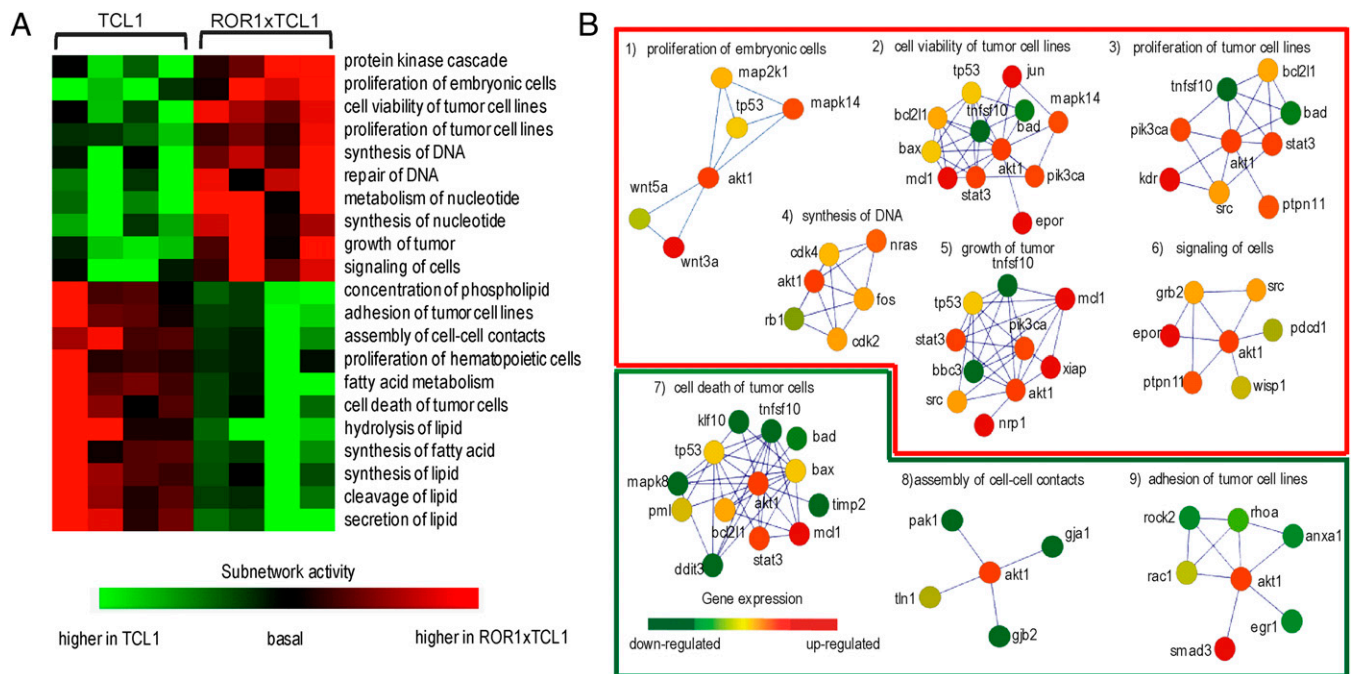
Fig. 3. Subnetwork analyses of the genes expressed by ROR1 × TCL1 leukemia cells versus TCL1 leukemia cells. (*A*) Heatmap depicting the 21 subnetworks most differentially expressed between CD5+B220^low splenic leukemia cells of four ROR1 × TCL1 Tg mice (*Right* four columns) and four TCL1 Tg mice (*Left* four columns), as indicated on the *Right*. The color of each block depicts the integrated expression levels of genes within each subnetwork (rows) as being up-regulated or down-regulated in ROR1 × TCL1 leukemia cells versus TCL1 leukemia cells, as indicated in the scale below. Expression values were standardized across the two pooled groups. (*B*) Illustration of representative subnetworks defined by AKT and its first neighbors within selected subnetworks that were up-regulated (1–6) or down-regulated (7–9) in ROR1 × TCL1 versus TCL1 leukemia cells. Nodes and links represent proteins and protein physical interactions, respectively. The color of each node scales with the change in gene expression as indicated in the scale (*Lower Left*). The associated cellular functions are indicated above each subnetwork.
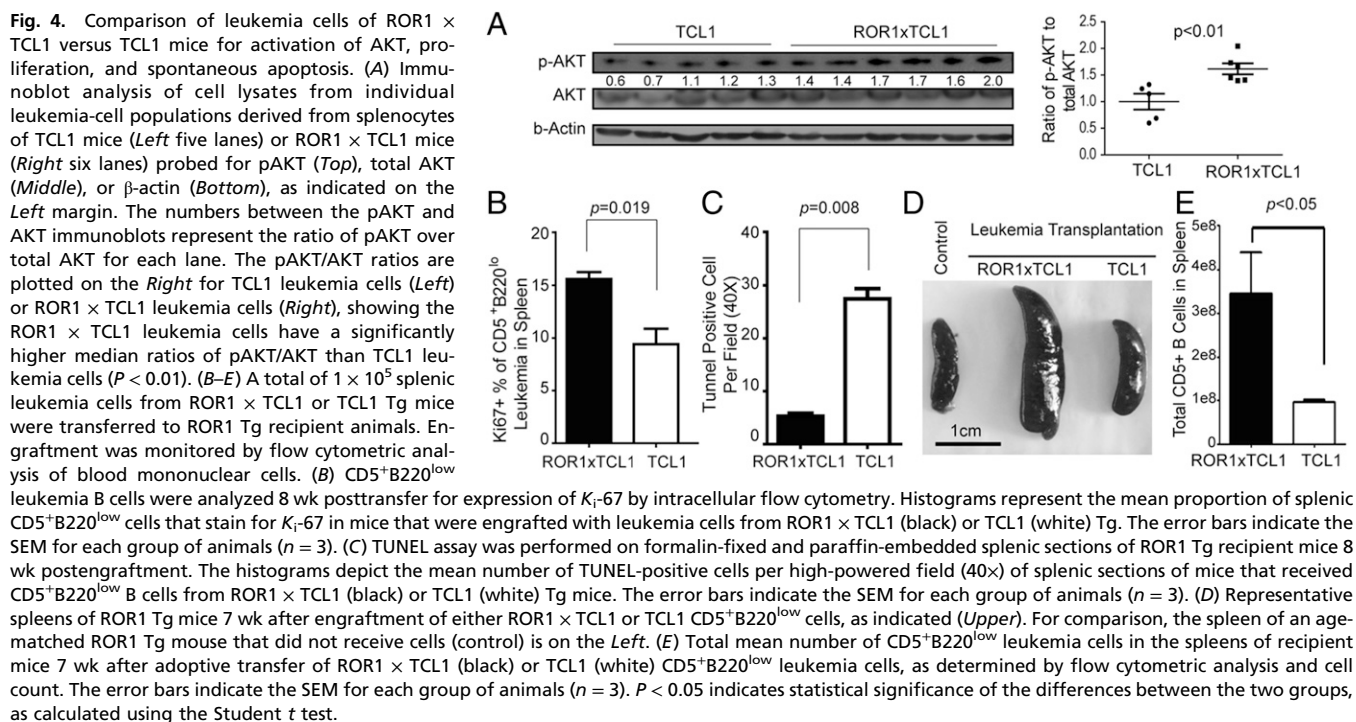
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splenocytes of mice engrafted with leukemia cells from ROR1 × TCL1 Tg mice had significantly lower proportions of cells undergoing apoptosis ($5\% \pm 0.6\%$, median = 5%, $n = 3$) than did the splenocytes of mice engrafted with leukemia from TCL1 Tg mice ($27\% \pm 1.9\%$, median = 25%, $n = 3$, $P < 0.01$), as assessed via terminal deoxynucleotidyl transferase nick end-labeling (TUNEL) staining of splenic tissue sections (Fig. 4C and Fig. S6). These data indicate that expression of ROR1 may promote $CD5^+B220^{low}$ B-cell proliferation and survival.

That such changes were associated with more aggressive disease was demonstrated in adoptive transfer studies on leukemia cells into syngeneic ROR1 Tg mice. For example, i.v. injection of 1×10^5 splenic leukemia B cells from ROR1 × TCL1 resulted in earlier and more marked splenomegaly than did injection of the same number of leukemia cells from TCL1 Tg mice (Fig. 4D). Moreover, 7 wk after adoptive transfer, animals engrafted with ROR1 × TCL1 leukemia cells had a 3.6-fold greater number of $CD5^+B220^{low}$ B cells in the spleen ($3.5 \pm 0.9 \times 10^8$, median = 3.3×10^8, $n = 3$) than did animals engrafted with TCL1 $CD5^+B220^{low}$ B cells ($9.6 \pm 0.6 \times 10^7$, median = 9.0×10^7, $n = 3$, $P < 0.05$) (Fig. 4E).
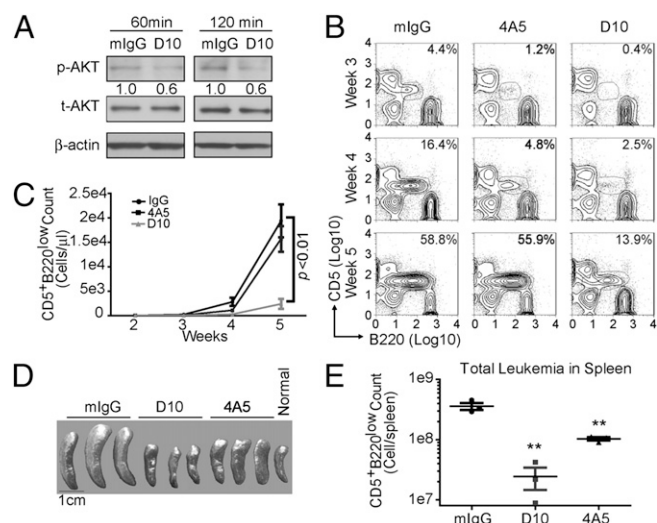
Treatment of ROR1 × TCL1 Leukemia with Anti-ROR1 mAbs. We examined the activity of two different mouse anti-human ROR1 mAbs, D10 and 4A5, which bind to distinct nonoverlapping epitopes of ROR1, as assessed in cross-blocking studies (Fig. S7A). We also noted that the binding affinity of D10 for ROR1 was eightfold lower (41 nM) than that of 4A5 (5 nM), as assessed by liquid phase antigen–antibody interactions in a kinetic exclusion assay (Fig. S7B). Nevertheless, recent studies found that D10 could inhibit $ROR1^+$ breast cancer metastasis in vivo (32). As with $ROR1^+$ breast cancer cells, the D10 mAb could induce rapid down-modulation of ROR1 on ROR1 × TCL1 leukemia cells in vitro at 37 °C, an effect that was less apparent with the 4A5 mAb (Fig. S7C). Furthermore, treatment of ROR1 × TCL1 leukemia cells with D10 in vitro resulted in reduced expression of pAKT within 1 h after addition of the antibody to the leukemia cells, an effect that was not apparent in control Ig-treated cells (Fig. 5A).

We treated ROR1 Tg mice engrafted with $CD5^+B220^{low}$ $ROR1^+$ leukemia cells with weekly i.v. injections of D10, 4A5, or control mouse IgG (mIgG), at 10 mg/kg. $CD5^+B220^{low}$ leukemia cells were detectable in the blood of mIgG-treated mice at 3 wk posttransfer, whereas they were not present in the ROR1 Tg mice that received D10, and were decreased in 4A5-treated animals (Fig. 5B). At 5 wk, mice given D10 had significantly fewer $CD5^+B220^{low}$ leukemia cells ($2.4 \pm 1.0 \times 10^3/\mu L$, $n = 3$) in the blood than mice that received mIgG ($2.0 \pm 0.3 \times 10^4/\mu L$, $n = 3$, $P = 0.03$) (Fig. 5C), whereas the number of leukemia cells in the blood of mice given 4A5 ($1.6 \pm 0.3 \times 10^4/\mu L$, $n = 3$, $P > 0.05$) was not significantly different from that of mice treated with mIgG.

In another experiment, we transferred fewer $CD5^+B220^{low}$ $ROR1^+$ B cells (5×10^4) and administered weekly doses of 10 mg/kg of either mAb or control mIgG, to better assess the difference in activity between D10 and 4A5. ROR1 Tg mice given mIgG had significantly larger spleens than age-matched ROR1 Tg littermates that did not receive $CD5^+B220^{low}ROR1^+$ B cells when the animals were killed 4 wk later (Fig. 5D). Mice that received $CD5^+B220^{low}ROR1^+$ B cells and that were treated with D10 had significantly smaller spleens than mice that were treated with mIgG or 4A5. The proportion of $CD5^+B220^{low}ROR1^+$ B cells in the spleen was determined by flow cytometric analysis and the total number of $CD5^+B220^{low}ROR1^+$ B cells per spleen was enumerated (Fig. 5E). Compared with the mIgG-treated mice that had $3.6 \pm 0.5 \times 10^8$ (median = 3.3×10^8, $n = 3$) leukemia cells per spleen, both D10-treated animals ($2.4 \pm 1.0 \times 10^7$, median = 2.2×10^7, $n = 3$, $P = 0.02$) and 4A5-treated mice ($1.0 \pm 0.1 \times 10^8$, median = 1.1×10^8, $n = 3$, $P = 0.006$) had significantly fewer $CD5^+B220^{low}ROR1^+$ B cells following administration of anti-ROR1 mAb. The number of $CD5^+B220^{low}$ $ROR1^+$ B cells in the spleen of D10-treated mice was also significantly lower than the number in the spleen of 4A5-treated animals ($P = 0.003$, $n = 3$). Although D10 was effective in inhibiting the engraftment of ROR1 × TCL1 leukemia cells, administration of either the D10 or 4A5 anti-ROR1 mAb had no effect on the endogenous nonleukemia ($CD5^-B220^{hi}ROR1^+$) B cells (Fig. S8A) or T cells (Fig. S8B) of the ROR1 Tg recipient mice. That the effect of D10 was due to its activity against

Fig. 4. Comparison of leukemia cells of ROR1 × TCL1 versus TCL1 mice for activation of AKT, proliferation, and spontaneous apoptosis. (A) Immunoblot analysis of cell lysates from individual leukemia-cell populations derived from splenocytes of TCL1 mice (Left five lanes) or ROR1 × TCL1 mice (Right six lanes) probed for pAKT (Top), total AKT (Middle), or β-actin (Bottom), as indicated on the Left margin. The numbers between the pAKT and AKT immunoblots represent the ratio of pAKT over total AKT for each lane. The pAKT/AKT ratios are plotted on the Right for TCL1 leukemia cells (Left) or ROR1 × TCL1 leukemia cells (Right), showing the ROR1 × TCL1 leukemia cells have a significantly higher median ratios of pAKT/AKT than TCL1 leukemia cells ($P < 0.01$). (B–E) A total of 1×10^5 splenic leukemia cells from ROR1 × TCL1 or TCL1 Tg mice were transferred to ROR1 Tg recipient animals. Engraftment was monitored by flow cytometric analysis of blood mononuclear cells. (B) $CD5^+B220^{low}$

leukemia B cells were analyzed 8 wk posttransfer for expression of K_i-67 by intracellular flow cytometry. Histograms represent the mean proportion of splenic $CD5^+B220^{low}$ cells that stain for K_i-67 in mice that were engrafted with leukemia cells from ROR1 × TCL1 (black) or TCL1 (white) Tg. The error bars indicate the SEM for each group of animals ($n = 3$). (C) TUNEL assay was performed on formalin-fixed and paraffin-embedded splenic sections of ROR1 Tg recipient mice 8 wk postengraftment. The histograms depict the mean number of TUNEL-positive cells per high-powered field (40×) of splenic sections of mice that received $CD5^+B220^{low}$ B cells from ROR1 × TCL1 (black) or TCL1 (white) Tg mice. The error bars indicate the SEM for each group of animals ($n = 3$). (D) Representative spleens of ROR1 Tg mice 7 wk after engraftment of either ROR1 × TCL1 or TCL1 $CD5^+B220^{low}$ cells, as indicated (Upper). For comparison, the spleen of an age-matched ROR1 Tg mouse that did not receive cells (control) is on the Left. (E) Total mean number of $CD5^+B220^{low}$ leukemia cells in the spleens of recipient mice 7 wk after adoptive transfer of ROR1 × TCL1 (black) or TCL1 (white) $CD5^+B220^{low}$ leukemia cells, as determined by flow cytometric analysis and cell count. The error bars indicate the SEM for each group of animals ($n = 3$). $P < 0.05$ indicates statistical significance of the differences between the two groups, as calculated using the Student t test.

Fig. 5. The anti-ROR1 mAb D10 inhibits engraftment of CD5+B220lowROR1+ leukemia cells in ROR1 Tg mice. (*A*) Lysates were prepared from CD5+B220lowROR1+ leukemia cells that previously were treated for 1 or 2 h with either 40 μg/mL of control IgG or D10 for immunoblot analysis with antibodies specific for pAKT (*Top* row), total AKT (*Middle* row), or β-actin (*Bottom* row). The numbers between the *Top* and *Middle* rows are the ratio of the density of pAKT divided by that of AKT, as in Fig. 4. (*B–E*) ROR1 Tg mice were engrafted with 5×10^5 CD5+B220lowROR1+ leukemia cells and then given weekly i.v. injections of antibody at 10 mg/kg. (*B*) The blood mononuclear cells of engrafted mice were monitored weekly for CD5+B220low leukemia cells by flow cytometry. Contour plots depict staining of lymphocytes, as determined by light scatter characteristics, with fluorochrome-conjugated mAbs specific for mouse CD5 (*y* axis) and mouse B220 on the abscissa at 3 wk (*Top* row), 4 wk (*Middle* row), or 5 wk (*Bottom* row) of engrafted mice that were treated with control IgG (*Left* column), 4A5 (*Center* column), or D10 (*Right* column). The percentages in the *Top Right* of each contour plot indicate the proportion of the blood mononuclear cells having CD5+B220low phenotype of the leukemia cells. (*C*) Indicated in the graph are the numbers of leukemia cells detected over time in mice treated with D10 (gray), 4A5 (black), or control mIgG (black). (*D*) Representative spleens of ROR1 Tg mice 4 wk after engraftment with 5×10^4 CD5+B220low B cells and treatment with either control IgG (*Left* three), 4A5 (*Center* three), or D10 (*Right* three) mAbs, as indicated above. For comparison, a representative spleen of a ROR1 Tg mouse that had not been engrafted is to the *Far Right* (marked normal). (*E*) Total number of CD5+B220low leukemia cells in spleens of recipient mice 4 wk after adoptive transfer of 5×10^4 CD5+B220low B cells that received weekly injections of 10 mg/kg of mIgG, D10, or 4A5 mAb, as determined by flow cytometric analysis and cell count. Each square represents the number of leukemia cells found in individual mice. The bars represent the mean number for each group ± SEM; $n = 3$; *$P < 0.05$, **$P < 0.01$, compared with the mIgG group, as calculated using the Student t test.

ROR1-expressing CLL cells was confirmed in studies on mice engrafted with TCL1 leukemia cells, in which there was no significant difference in the spleen size or median numbers of leukemia cells found in the spleens of engrafted animals following treatment with either D10 or mIgG (Fig. S9 *A–D*).

Discussion

We generated Tg mice that express human ROR1 under the control of the mouse IgH promoter/enhancer, providing for B-cell–restricted expression of human ROR1. Constitutive B-cell expression of human ROR1 was conducive to developing clonal expansions of CD5+B220low B cells, which were not observed in littermates without the ROR1 transgene. Animals that developed such expansions had lymphocytosis and splenomegaly due to a lymphoproliferative disease that could be transferred without preconditioning to syngeneic mice, which in turn also developed a lymphoproliferative disease sharing the same clonal Ig gene rearrangement(s) as that of adoptively transferred CD5+

B220low B cells. As such, this study demonstrates that constitutive expression of ROR1 can lead to development of a leukemia that shares many phenotypic features in common with human CLL.

TCL1 mice also develop a CD5+B220low B-cell leukemia, but at earlier ages, generally around 7–9 mo. However, in contrast to human CLL, we found that such CD5+B220low leukemia B cells lacked expression of mouse ROR1, indicating that ROR1 is not necessary for leukemogenesis in this model. In human CLL cells and leukemia cells that developed in ROR1 × TCL1 Tg mice, we found that ROR1 could complex with TCL1, suggesting that it might influence the biology of TCL1, a known coactivator of AKT (27–31). Consistent with this notion, we found that ROR1 × TCL1 Tg mice develop leukemia at significantly younger ages than TCL1 Tg mice.

Network analyses of transcriptome data revealed higher expression of several subnetworks involving Akt1 that are significantly associated with enhanced cell proliferation and lower expression of several subnetworks associated with cell death, in ROR1 × TCL1 leukemia cells relative to that in TCL1 leukemia cells. We also observed statistically significant transcriptional upregulation of the canonical AKT signaling pathway involving 11 of its 18 genes, as well as higher levels of pAKT in leukemia cells of ROR1 × TCL1 mice relative to those of TCL1 mice. Consistent with this, we found that the ROR1 × TCL1 leukemia cells have enhanced rates of proliferation and reduced levels of spontaneous apoptosis than TCL1 leukemia cells, accounting for the more aggressive nature of ROR1 × TCL1 leukemia than TCL1 leukemia upon adoptive transfer into syngeneic recipient mice.

Expression of ROR1 in solid tumors was associated with enhanced activation of AKT, which in turn induces expression of genes that can enhance resistance to apoptosis and/or promote tumor cell growth in breast (10) or lung cancer (12). Moreover, we found that expression of ROR1 in any one of a variety of different human cancers was associated with higher levels of phosphorylated AKT than cancers that lacked detectable ROR1 (11). As embryonic cells and solid-tissue cancers can express TCL1 (33, 34), it is conceivable that ROR1 also might be associated with higher levels of pAKT via its interaction with TCL1, which, in prior studies, has been demonstrated to be a coactivator of AKT (27–31). However, ROR1 also might interact with proteins other than TCL1 to influence activation of AKT. Relevant to consider are studies on mice made genetically deficient in *TCL1* that lacked development of T and B cells, but had otherwise normal organ development (35). As such, TCL1 does not appear to be necessary for the development and/or activation of AKT in most other normal tissues other than in lymphoid tissues, raising the prospect that ROR1 might complex with other proteins in other tissues to enhance activation of AKT. Nevertheless, as shown in this study, the constitutive coexpression of both ROR1 and TCL1 can accelerate leukemogenesis and lead to enhanced activation of AKT over that observed in leukemia cells that express TCL1 alone.

We also demonstrated that antibodies specific for ROR1, such as D10, could cause down-modulation of ROR1, which in turn was associated with down-modulation of pAKT. The mechanism contributing to this down-modulation is unknown. In any case, it does appear such down-modulation is associated with reduced levels of pAKT, as has been shown for other ROR1+ cancers in which expression of ROR1 was repressed with shRNA (10, 12).

Conceivably, the down-modulation of ROR1 is responsible for the effect that this mAb has on the engraftment of ROR1 × TCL1 leukemia cells in vivo, a property that is not shared by all mAbs that bind to ROR1. Even though 4A5 binds to ROR1 with nearly an order of magnitude higher affinity than D10, this mAb is less effective in inhibiting the growth of ROR1 × TCL1 leukemia cells in vivo. It appears unlikely that this is due to differences in antibody subclass, as the heavy chain constant region isotype of D10 or 4A5 is IgG2a or IgG2b, respectively, each of which could direct antibody-dependent cellular cytotoxicity (36–38). Instead we hypothesize that the activity of each mAb also may be influenced by how each mAb binds to ROR1, which

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could be influenced by the epitope that each mAb binds. This appeared likely to be the case when we examined human antisera generated against ROR1 in patients who were immunized with their own leukemia cells; whereas a subset of such patients made anti-ROR1 autoantibodies, only a smaller subset of such patients developed anti-ROR1 antibodies that appeared capable of blocking interactions between ROR1 and its identified ligand Wnt5a (1).

Collectively, this study provides insight into the function of ROR1 in CLL, in which it might complex with TCL1 and provide for higher activation levels of AKT, potentially leading to changes in gene expression that promote cell proliferation and reduce the rate of spontaneous apoptosis. Antibodies targeting ROR1, such as D10, have the potential to offset the growth promoting activity of ROR1 in cells that express both ROR1 and TCL1, such as human CLL. As such, ROR1 appears to be an excellent target for development of novel therapies for patients with this disease or other cancers that also express this oncoembryonic protein.

Materials and Methods

Transgenic Mice. A 2.8-kb fragment possessing the entire human *ROR1* coding region was cloned into the EcoRV and XbaI sites of the pBSVE6BK (pEμ) plasmid containing a mouse V$_H$ promoter and the IgH-μ-enhancer, along with the 3′ untranslated region and the poly(A) site of the human β-globin gene (Fig. S1A). The construct containing *ROR1* free from vector sequences was injected into fertilized oocytes from C57BL/6 animals. Mice were screened for the presence of the transgene by PCR and flow cytometry. One founder was obtained and bred. Transgenic offspring from these founders were studied and compared with nontransgenic siblings raised under identical conditions. Genotyping was performed on genomic DNA by PCR. Eμ-TCL1 transgenic (TCL1-Tg) mice were backcrossed more than 10 generations

onto the C57BL/6 background, as described (26). All Tg mice were maintained by mating with C57BL/6 animals, except for ROR1 × TCL1 mice, which were generated by mating ROR1 Tg mice with TCL1 Tg mice. All mice were housed and bred in a specific pathogen-free animal facility, treated in accordance with the National Institutes of Health guidelines for care and use of laboratory animals and in accordance with animal protocols approved by University of California San Diego's Animal Care Program. Survival data were obtained by observing cohorts of 26 mice of each genotype. Mice were included in the analysis after spontaneous death due to advanced disease or when euthanized when they developed morbidity due to advanced leukemia, which was associated with lethargy, impaired mobility, shallow or labored breathing, massive splenomegaly, and lymphocytosis due to clonal CD5$^+$B220^{low} B cells.

CLL Patient Samples. Samples were collected from patients evaluated at University of California (UCSD) San Diego Moores Cancer Center after they provided written informed consent on a protocol approved by the Institutional Review Board (IRB) of UCSD (IRB approval number 080918), in accordance with the Declaration of Helsinki. All patients fulfilled diagnostic criteria for CLL.

A detailed description of the reagents, cellular assays, adoptive transfer, protein analysis, microarray, subnetwork analysis, GSEA, animal studies, and statistics are available in *SI Materials and Methods*.

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