T Cell Factor 1 Regulates Thymocyte Survival via a ROR γt-Dependent Pathway

Ruiqing Wang, Huimin Xie, Zhaofeng Huang, Jian Ma, Xianfeng Fang, Yan Ding and Zuoming Sun

*J Immunol* 2011; 187:5964-5973; Prepublished online 28 October 2011;
doi: 10.4049/jimmunol.1101205
http://www.jimmunol.org/content/187/11/5964

References

This article cites 38 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/187/11/5964.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
T Cell Factor 1 Regulates Thymocyte Survival via a RORγt-Dependent Pathway

Ruiqing Wang,*† Huimin Xie,‡ Zhaofeng Huang,§ Jian Ma,* Xianfeng Fang,* Yan Ding,* and Zuoming Sun*

Survival of CD4+/CD8+ double-positive (DP) thymocytes plays a critical role in shaping the peripheral T cell repertoire. However, the mechanisms responsible for the regulation of DP thymocyte lifespan remain poorly understood. In this work, we demonstrate that T cell factor (TCF)-1 regulates DP thymocyte survival by upregulating RORγt. Microarray analysis revealed that RORγt was significantly downregulated in TCF-1−/− thymocytes that underwent accelerated apoptosis, whereas RORγt was greatly upregulated in thymocytes that had enhanced survival due to transgenic expression of a stabilized β-catenin (β-cat18), a TCF-1 activator. Both TCF-1−/− and RORγt−/− DP thymocytes underwent similar accelerated apoptosis. Forced expression of RORγt successfully rescued TCF-1−/− DP thymocytes from apoptosis, whereas ectopically expressed TCF-1 was not able to rescue the defective T cell development because of the lack of RORγt-supported survival. Furthermore, activation of TCF-1 by stabilized β-catenin was able to enhance DP thymocyte survival only in the presence of RORγt, indicating that RORγt acts downstream of TCF-1 in the regulation of DP thymocyte survival. Moreover, β-catenin/TCF-1 directly interacted with the RORγt promoter region and stimulated its activity. Therefore, our data demonstrated that TCF-1 enhances DP thymocyte survival through transcriptional upregulation of RORγt, which we previously showed is an essential prosurvival molecule for DP thymocytes. The Journal of Immunology, 2011, 187: 5964–5973.

Survival of CD4+/CD8+ double-positive (DP) thymocytes is critical for T cell development, because the lifespan of thymocytes determines their chances to be either positively selected and mature into CD4+ or CD8+ cells or negatively selected and undergo apoptosis (1, 2). TCR α-chain rearrangement occurs during the DP stage, and the rearranged α-chain then pairs with the previously generated TCRβ-chain to form a mature TCR. If a TCR fails positive selection, the DP thymocyte has the opportunity to start another round of TCRα-chain rearrangement until the cell reaches the end of its lifespan and apoptosis initiates. These multiple rounds of TCRα-chain rearrangement in DP thymocytes significantly increase the possibility of generating a functional TCR and, thus, increase the opportunity for successful positive selection (3). Thus, the lifespan of DP thymocytes limits the progression of TCRα-chain rearrangement and positive selection (2). However, little is known about the survival mechanisms that prevent apoptosis of DP cells prior to completion of the selection process.

We (4) and other investigators (2, 5) identified retinoic acid receptor-related orphan receptor γt (RORγt) as a critical molecule in controlling the survival of DP thymocytes. We showed that RORγt recruited the steroid receptor coactivators (SRC) to maintain thymocyte survival by upregulating the prosurvival molecule Bcl-xL (6). Bcl-xL is required for DP thymocyte survival, because deletion of the Bcl-xL gene leads to extensive apoptosis of DP thymocytes (7), and overexpression of Bcl-xL extends thymocyte survival (4, 8, 9). Although we identified an interacting protein (SRC) and the downstream molecule (Bcl-xL) implicated in RORγt-regulated thymocyte survival, the upstream signaling pathways that regulate RORγt-mediated thymocyte survival remain to be elucidated.

The transcription factor T cell factor (TCF)-1 is also essential for DP thymocyte survival, because deletion of TCF-1 results in massive apoptosis of DP thymocytes (1, 10). TCF-1 is usually kept inactive by association with a transcriptional repressor (Groucho-related gene in mouse, Groucho in Drosophila, or TLE in humans), which inhibits the expression of TCF-1 target genes (11). In response to Wnt signals, glycogen synthase kinase-3β (GSK-3β), which phosphorylates and induces degradation of β-catenin (12), is inactivated. Inactivation of GSK-3β leads to the stabilization and accumulation of β-catenin, a transcriptional coactivator for TCF-1. The accumulated β-catenin then replaces the transcriptional repressor GRG, resulting in the activation of TCF-1. Binding to β-catenin is required for TCF-1–mediated thymocyte survival, because a mutant TCF-1, incapable of binding to β-catenin, could not support DP thymocyte survival (10). We also demonstrated that a stabilized, constitutively active β-catenin enhanced DP thymocyte survival by upregulating Bcl-xL levels (6). Therefore, both RORγt and TCF-1 regulate thymocyte survival by specific upregulation of Bcl-xL in DP thymocytes (1), which raises the possibility that TCF-1 and RORγt...
might be involved in the same pathway regulating DP thymocyte survival. In this study, we used TCF-1–deficient (TCF-1−/−), RORγt-deficient (RORγt−/−), and transgenic mice that expressed a constitutively active β-catenin (β-catE5) to demonstrate that the β-catenin/TCF-1 pathway is upstream of RORγt in the regulation of DP thymocyte survival. We showed that RORγt expression was significantly reduced in the absence of TCF-1 but was greatly upregulated in mice expressing constitutively active β-catenin, a TCF-1 coactivator. In addition, forced expression of RORγt restored Bcl-2 expression and rescued the survival of DP thymocytes from TCF-1−/− mice. In contrast, neither transgenic expression of β-catenin nor forced expression of TCF-1 rescued RORγt-deficient thymocytes from apoptosis. Moreover, we found that β-catenin/TCF-1 directly activates RORγt transcription by binding to its proximal regulatory region. Thus, our studies demonstrated that the β-catenin/TCF-1 pathway enhances DP thymocyte survival in a RORγt-dependent manner, and RORγt is likely a direct target of β-catenin/TCF-1 in thymocytes.

Materials and Methods

Mice

Transgenic mice (C57BL/6 background) that expressed stabilized β-catenin were generated, as described previously (13), and C57BL/6 TCF-1−/− mice were obtained from Dr. H. Clevers (Hubrecht Laboratory, Center for Biomedical Genetics, Utrecht, The Netherlands). The RORγt−/− mouse strain was generated, as previously described (4). All mice were bred and housed under specific pathogen-free conditions in the Animal Research Center (City of Hope).

Plasmids and cell lines

DNA fragments containing different regions of the RORγt promoter were obtained by PCR using mouse genomic DNA as a template and were cloned into pGL3 basic (Promega). Wild-type (WT) and stabilized β-catenin expression plasmids were provided by Drs. F. McCormick and O. Tetsu (University of California, San Francisco, CA). The retroviral vector MIGR (a gift from Dr. Warren S. Pear, University of Pennsylvania, Philadelphia, PA) was described previously (14). The MIGR-RORγt, MIGR–TCF-1, and MIGR–active-β-catenin vectors were constructed by subcloning the cDNA fragments into the XhoI and EcoRI, BglII and EcoRI, and Hpal sites of MIGR1, respectively, and sequenced. Retrovirus-producing cell line Phoenix was kindly offered by Dr. Takahiro Maeda (City of Hope).

Flow cytometry and cell sorting

Thymi of 6–8-wk-old mice were harvested, and single-cell suspensions of thymocytes were prepared and stained with fluorochrome-conjugated Abs. Cells and other reagents were purchased as follows: allophycocyanin–anti-CD4 (catalog No. 17-0041-81), PE–anti-CD8 (catalog No. 25-0081-81), and PE–anti-CD25 (catalog No. 12-0251-82) Abs were from eBioscience; PE–Annexin V (catalog No. 556621), 7-aminoactinomycin D (7-AAD; catalog No. 51-69891E), and PE–anti-Thy1.2 (Clone 30-H12, catalog No. 533014) and FITC–anti-CD4 (catalog No. 553133) Abs were from BD Pharmingen. FACS assays were performed using a FACSCantoII (BD Biosciences) and analyzed with FlowJo software (Tree Star). Electronic cell sorting was done on a FACSariaII (BD Biosciences).

Apoptosis assays

Thymocytes were freshly isolated from WT, TCF-1−/−, or RORγt−/− mice and cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine (Life Technologies), and 50 μM 2-ME (Sigma-Aldrich) at 1 × 10⁶ cells/ml. Thymocytes were treated or not with 10−7 M dexamethasone (Dex) for the indicated times at 37°C in 5% CO₂. Dead cells were detected by Annexin V-PE and 7-AAD staining, as described previously (13).

Western blot analysis

Thymocytes isolated from mice of each genotype were lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 4 mM KCl, 1 mM MgCl₂, 1 mM Na₂VO₄, 1% Nonidet P-40 (NP-40), and protease inhibitors). Protein from the thymocyte extracts (15 μg) was fractionated on 10% SDS-polyacrylamide gels and transferred to Immobilon FL PVDF Membrane (Millipore). Membranes were blocked in blocking buffer (LI-COR) and then incubated with primary Ab for 1 h to overnight at 4°C. Membranes were washed in TBST (10 mM Tris-base, 0.15 M NaCl, 0.05% MgCl₂, 0.1% Tween 20, 0.01% benzethonium chloride [pH 7.4]) and probed with anti-mouse or rat IRDye 680 (LI-COR) for 1 h at room temperature. After washing in TBST, the proteins were detected by an Odyssey Infrared Imaging System (LI-COR). Western primary Abs included anti-RORγt (clone H-190, catalog No. 28559) and anti-actin Abs (catalog No. SC-8422) from Santa Cruz Biotechnology, anti–Bcl-2 (clone 54H6, catalog No. 2764) and anti–TCF-1 (clone C63D9, catalog No. 2203S) from Cell Signaling Technology (Beverly, MA), and anti–β-catenin Abs (catalog No. 610154) from BD Biosciences.

Cell culture, transient transfection, and reporter assay

293T cells were cultured in DMEM supplemented with 10% FBS, 2 mM glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells (2 × 10⁵ in each well of a 24-well plate) were transfected with the reporter plasmid (100 ng), pSV40-Renilla luciferase vector (50 ng), and expression vectors (0.5 μg) by the calcium phosphate-precipitation method. The total amount of transfected DNA was kept constant by adjusting the amount of the empty vector. Cells were collected after 24 h and lysed in 200 μl of luciferase buffer (137 mM NaCl, 50 mM Tris-Cl, 0.5% NP-40); luciferase activities were measured by the Dual Luciferase system, according to the manufacturer’s instructions (Promega), and normalized against Renilla luciferase activities. “Folds of stimulation” represents normalized luciferase activity divided by the result of reporter-only groups.

Quantitative real-time PCR

Freshly isolated or electronically sorted thymocytes were lysed and homogenized over Quanishereder columns (Qiagen). Total RNA was extracted by RNeasy Plus Mini Kit (Qiagen), according to the manufacturer’s instructions, and reverse transcribed with Superscript II first-strand cDNA synthesis system with oligo(dT) (Invitrogen), according to the manufacturer’s guide. Quantitative real-time PCR (qRT-PCR) was performed using SsoFast EvaGreen Supermix (Bio-Rad) in a CFX96 Real-Time PCR Detection System (Bio-Rad), according to the manufacturer’s protocol. The amplification efficiency of all primers has been tested, and the optimized conditions were used in all qRT-PCR reactions. The amplification of the housekeeping gene GAPDH was used to normalize the DNA content in templates. Primers used were as follows: RORγt, 5′-CCGCTTAGAG-GCGCTTCAC-3′ and 5′-TGGCAAGTGGAGCAATCAACA-3′; TCF-1, 5′-AGACAATCCTGAGAAGCTT-3′ and 5′-GTGGAAGCTCTGAAG-TCGATGG-3′; Bcl-2, 5′-AAGGACTAGTAAGAATGCTTCTG-3′ and 5′-CTGACCATGGGACACTGAGTTG-3′; and GAPDH, 5′-CTGGTGAAG-GTGCTGGTGAAC-3′ and 5′-CATGATTGGTGAATGTCAGG-3′.

Coculture and transduction of thymocytes on OP9-DL1 stromal cells

Thymocytes were allowed to differentiate on OP9-DL1 cells (gift from Dr. Timothy P. Bender, University of Virginia Health System, Charlottesville, VA) (15–17). Briefly, electronically sorted double-negative thymocytes (Thy1.2+CD4⁻/⁻) were cultured with OP9-DL1 cells in flat-bottom 24-well culture plates with OSM (MEM (MEM medium; Invitrogen Life Technologies) supplemented with 20% FBS, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine (Invitrogen Life Technologies), and 5 ng/ml recombinant murine IL-7 (PeproTech). The following day, cocultures were transduced by spinfection (2500 rpm, 90 min) using retroviral supernatants in the presence of 5 μg/ml polybrene (Sigma-Aldrich). Retroviral supernatants were produced by transfection of Ca²⁺/PO₄ transfection of Phoenix cells with the MIGR1–expression vector. Seventy-two hours posttransduction, cocultures were harvested for flow cytometry.

Chromatin immunoprecipitation

Thymocyte suspension (10⁶ cells) was incubated (10 min, room temperature) with 1% formaldehyde to cross-link proteins with chromatin. Glycine (125 mM) was added to stop the cross-linking reaction. Cells were collected, washed with cold PBS that contained protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin and leupeptin), and lysed with SDS lysis buffer (1% SDS, 5 mM EDTA [ethylene-diamine tetra-acetic acid]) with protease inhibitors for 20 min on ice. Cell lysates were sonicated to shear genomic DNA into fragments 200–1000 bp and centrifuged (15 min, 13,000 rpm, 4°C). Supernatant was collected and diluted with chromatin immuno-
Results

**RORγt expression is dependent on TCF-1 in thymocytes**

Our previous studies demonstrated that activation of TCF-1 by transgenic expression of a stabilized β-catenin (β-cat\(^ {T 2 8} \)) enhances thymocyte survival through upregulation of the prosurvival factor Bcl-x\(_L\) (6). In contrast, deletion of TCF-1 leads to thymocyte apoptosis due to downregulation of Bcl-x\(_L\) (10). To identify downstream molecules responsible for TCF-1 pathway-regulated thymocyte survival, we performed microarray analysis on WT, TCF-1\(^ {−/−} \), and β-cat\(^ {T 2 8} \) thymocytes. Gene expression was first compared between TCF-1\(^ {−/−} \) and WT thymocytes (Fig. 1A, x-axis). Most genes were clustered near “1,” indicating no significant change in expression levels compared with WT, whereas positive or negative numbers indicate the folds of up- or downregulation in log\(_2\) scale. CD4 and Bcl-x\(_L\), which are known to be expressed at much lower levels in TCF-1\(^ {−/−} \) thymocytes (6, 18), were indeed found to be downregulated in the microarray results. Gene expression was also compared between WT and β-cat\(^ {T 2 8} \) thymocytes (Fig. 1A, y-axis). Because β-catenin activates TCF-1, genes that were downregulated in TCF-1\(^ {−/−} \) mice were expected to be upregulated in β-cat\(^ {T 2 8} \) mice. As expected, both CD4 and Bcl-x\(_L\) were upregulated in β-cat\(^ {T 2 8} \) thymocytes compared with WT counterparts. The opposite change in the expression level of both CD4 and Bcl-x\(_L\) genes in TCF-deficient versus β-cat\(^ {T 2 8} \) thymocytes assured us that the results of our microarray analysis were reliable.

One of the major problems with microarray assay is identification of too many potential target genes. However, because we had gene-expression profiles from both TCF-1 knockout (TCF-1\(^ {−/−} \)) and TCF-1\(^ {+/+} \) thymocytes, we performed microarray analysis on WT, TCF-1\(^ {−/−} \), and β-cat\(^ {T 2 8} \) thymocytes. Gene expression was then compared between TCF-1\(^ {−/−} \) and WT (horizontal axis) and TCF-1\(^ {−/−} \) versus WT (vertical axis) comparisons are shown in the dot plot. Ninety-three genes downregulated in TCF-1\(^ {−/−} \) (threshold fold of change = 3.0) but upregulated in β-cat\(^ {T 2 8} \) (threshold fold of change = 1.7) thymocytes were subjected to IPA analysis of Ingenuity system. Under the biofunction categories of “cellular death” and “cellular development,” four genes (Bcl-x\(_L\), CD4, Fas, and RORγt) (annotated) that had the smallest \( p \) value were selected to continue searching for the possible TCF-1 targets. B, RORγt RNA expression is decreased in TCF-1\(^ {−/−} \) thymocytes and increased in thymocytes in β-cat\(^ {T 2 8} \) mice. Total RNA was purified from thymocytes of WT, TCF-1\(^ {−/−} \), RORγt\(^ {−/−} \), and β-cat\(^ {T 2 8} \) mice, and cDNA obtained from reverse-transcription was used as template for qRT-PCR detecting RORγt (left panel) and TCF-1 (right panel). Data are presented as mean ± SD and are representative of at least three separate experiments. C, Reduced RORγt protein levels were detected in thymocytes of TCF-1\(^ {−/−} \) mice. Western blot analysis of TCF-1 (top panel) and RORγt (middle panel) levels in thymocytes obtained from WT, RORγt\(^ {−/−} \) and TCF-1\(^ {−/−} \) mice. Data are representative of four independent experiments. D, TCF-1 upregulation occurs earlier than that of RORγt during T cell development. Thymocytes from five WT C57/B6 mice were isolated and stained with Abs against CD4, CD8, CD25, and CD44. The four stages of DN thymocytes were sorted based on the following gating: DN1, CD4\(^ +\) CD8\(^ −\) CD25\(^ −\) CD44\(^ +\); DN2, CD4\(^ +\) CD8\(^ −\) CD25\(^ +\) CD44\(^ +\); DN3, CD4\(^ +\) CD8\(^ +\) CD25\(^ −\) CD44\(^ +\); and DN4, CD4\(^ +\) CD8\(^ +\) CD25\(^ +\) CD44\(^ −\). TCF-1 (top panel) and RORγt (bottom panel) RNA levels of the sorted subsets of DN thymocytes were determined by qRT-PCR. Data are presented as mean ± SD (\( n = 3 \)) and are representative of three separate experiments. *\( p < 0.05 \), **\( p < 0.01 \).
and TCF-1-activated (β-caten16) conditions that had opposite effects on thymocyte survival. Therefore, we focused on those genes that were downregulated in TCF-1−/− thymocytes, but upregulated in β-caten18 thymocytes, because these genes are potentially positively regulated by TCF-1 activated by β-catenin. A total of 93 genes was found to fall into this category, among which Fas and Bcl-xL were repeatedly identified as apoptotic genes by Ingenuity IPA pathway software searching for the candidates involved in “cellular death” and “cellular development” with the smallest p value. The appearance of the genes Bcl-xL and Fas is not surprising, because both have been implicated in the regulation of cell survival by β-catenin/TCF-1 (6, 18–20). However, the gene RORγt, thymus-specific isoform of RORγt, elicited a great deal of interest, because our previous studies demonstrated that RORγt, similar to TCF-1, regulates DP thymocyte survival (4, 6). We performed qRT-PCR and Western blot to determine whether RORγt was downregulated in the absence of TCF-1. As shown in Fig. 1B, both RNA and protein levels of RORγt were significantly reduced in TCF-1-deficient thymocytes (we noted that the decrease in RORγt protein levels reflected by Western blot was not as striking as the reduction of RORγt RNA levels, which could be caused by differential turnover between mRNA and protein). In contrast, TCF-1 mRNA (Fig. 1B, right panel) and protein (Fig. 1C) levels were not reduced but were slightly increased in the absence of RORγt. These data suggested that RORγt expression is dependent on TCF-1, whereas TCF-1 levels are not affected by the absence of RORγt. Furthermore, contrary to the reduction of RORγt in TCF-1−/− thymocytes, the RORγt RNA level (Fig. 1B) was increased in β-caten18 thymocytes compared with WT counterparts, indicating that activation of the TCF-1 pathway by β-catenin leads to stimulation of RORγt expression. Thus, it appears that RORγt expression is regulated by the β-catenin/TCF-1 pathway in the thymocytes.

The expression and activity of transcription factors during T cell development are dynamically controlled. If TCF-1 regulates the expression of RORγt during thymocyte development as our microarray data indicated, we would expect the expression and/or activation of TCF-1 to occur prior to that of RORγt. To test this idea, we determined the expression time course of TCF-1 and RORγt in the four subsets of DN and DP populations in T cell development (Fig. 1D). We found that TCF-1 expression starts to increase significantly as early as the DN2 stage and peaks at the DP stage (Fig. 1D, top panel), whereas RORγt expression is not detectable until DN4 (Fig. 1D, bottom panel) and reaches the maximal level at DP as well. The observation that thymocytes start to express RORγt when the TCF-1 level is already relatively high is consistent with the possibility that, genetically, TCF-1 functions upstream of RORγt during T cell development and is required for upregulation of RORγt.

**Both TCF-1−/− and RORγt−/− thymocytes undergo similar apoptosis and downregulation of Bcl-xL**

To study the relationship between TCF-1 and RORγt-regulated thymocyte survival, we compared apoptosis kinetics of DP thymocytes from TCF-1−/− and RORγt−/− mice. Apoptosis was detected using Annexin V staining at various time points after thymocytes were cultured in medium in the absence (spontaneous apoptosis, Fig. 2A) or presence of Dex (Dex-induced apoptosis, Fig. 2B). DP thymocytes that lacked either TCF-1 or RORγt underwent accelerated apoptosis compared with WT cells in both spontaneous and Dex-induced cell death conditions, consistent with our previous findings and those of other groups (4, 10). In

![FIGURE 2. Thymocytes from TCF-1−/− and RORγt−/− mice undergo similar apoptosis. A. Accelerated spontaneous apoptosis of TCF-1−/− and RORγt−/− thymocytes. Thymocytes obtained from WT, TCF-1−/−, and RORγt−/− mice were cultured in RPMI 1640 medium for the indicated times. Apoptotic cells were detected by Annexin V and 7-AAD staining, and Annexin V and 7-AAD DN thymocytes were counted as live cells. B. Accelerated glucocorticoid-induced thymocyte apoptosis of TCF-1−/− and RORγt−/− thymocytes. Thymocytes obtained from WT, TCF-1−/−, and RORγt−/− mice were treated with 10−7 M Dex for the indicated times. Apoptotic cells were detected with Annexin V and 7-AAD staining, and the percentage of live cells was determined. Data in A and B represent mean percentage of live cells ± SD from at least five mice per group. C. Bcl-xL mRNA expression is reduced in thymocytes from both TCF-1−/− and RORγt−/− mice. Total RNA was purified from thymocytes of WT, TCF-1−/−, RORγt−/−, and β-caten18 mice, and cDNA obtained from reverse-transcription was used as template for qRT-PCR detecting Bcl-xL. D. Bcl-xL protein expression is reduced in thymocytes from both TCF-1−/− and RORγt−/− mice. Thymocytes from WT, RORγt−/−, and TCF-1−/− mice were subjected to Western blot analysis determining the protein levels of Bcl-xL (top panel), β-actin expression served as a loading control (bottom panel). Data are representative of four independent experiments.]
addition, both types of DP thymocytes underwent apoptosis at quite similar speed and scale. The resemblance of thymocyte apoptosis status in the absence of either factor is indicative of sharing of the same molecular pathway by the two factors in the regulation of thymocyte survival. We further examined expression of Bcl-xL, a critical prosurvival molecule for DP thymocytes (6, 7). Indeed, as reported before, both Bcl-xL mRNA (Fig. 2C) and protein (Fig. 2D) levels were significantly lower in TCF-1−/− and RORγt−/− cells compared with WT cells. Therefore, it appears that, functionally, TCF-1 and RORγt are involved in the same Bcl-xL–mediated DP thymocyte survival–regulation pathway. These data, combined with the reduction of RORγt in TCF-1−/− thymocytes, are indicative of a genetic axis “β-catenin/TCF-1 → RORγt → Bcl-xL” in the regulation of DP thymocyte survival.

β-catenin–enhanced thymocyte survival is dependent on RORγt

We started to prove the existence of the axis by answering the first question: Is RORγt required for β-catenin–mediated thymocyte survival enhancement? Previously, we demonstrated that transgenic expression of a constitutively active β-catenin (β-catTg) enhanced thymocyte survival (6). To test whether the enhanced thymocyte survival is dependent on RORγt, we crossed β-catTg mice with RORγt+/− mice, evaluated the thymocyte apoptosis status, and found that β-catTg/RORγt−/− thymocytes even lost the capability to survive as well as their WT counterparts (Fig. 3A). In fact, they underwent accelerated apoptosis as rapidly as did RORγt−/− thymocytes, suggesting that the enhanced DP thymocyte survival caused by overexpression of β-catenin is dependent on RORγt. Consistent with the observed accelerated apoptosis, β-catTg/RORγt−/− thymocytes had lower levels of Bcl-xL expression compared with WT or β-catTg cells (Fig. 3B), indicating that β-catenin–mediated upregulation of Bcl-xL is RORγt dependent as well. Taken together, these results indicated that enhancement of thymocyte survival by β-catenin, the TCF-1 activator, requires RORγt.

**Forced expression of RORγt in TCF-1−/− thymocytes restores Bcl-xL expression and rescues DP thymocyte survival**

To further prove the role of RORγt in TCF-1–regulated DP thymocyte survival, the crucial step would be to determine whether the “add-back” of RORγt is able to restore the expression of Bcl-xL and rescue DP thymocyte survival in TCF-1−/− thymocytes. To accomplish this, we used an in vitro differentiation system (IVD) that involves coculture of DN thymocytes with OP9 stromal cells that express the Notch ligand, Delta-like 1 (OP9-DL1) (15–17). To test the performance of the IVD system, we first evaluated whether WT thymocytes can normally differentiate, and in addition, whether the add-back of RORγt to RORγt-deficient thymocytes is able to remove the developmental blockade shown by our previous work (4). Fig. 4A shows a typical IVD experiment with WT cells. Sorted DN thymocytes (Fig. 4A, left panel) were cocultured with either stromal cells alone (Fig. 4A, middle panel) or with empty viruses (MIGR) that did not express any protein of interest (Fig. 4A, right panel). The appearance of >40% of DP cells indicated that our IVD system is able to support thymocyte development in vitro, and additionally, it seemed that retroviral transduction did not disturb this differentiation process, because the distribution of all four subgroups of thymocytes (DN, DP, CD4+, and CD8+) in nontreated and MIGR-treated thymocyte groups was quite similar. As we expected, most of the DN cells from RORγt−/− mice either remained in the DN subset or accumulated in the CD8+ immature single positive subset (TCRβlo, data not shown) (Fig. 4B, far left panel) when treated with empty viruses (Fig. 4B, second panel). Importantly, forced expression of RORγt rescued thymocyte development, indicated by the appearance of 50% DP thymocyte population (Fig. 4B, far right panel), suggesting that retrovirus-expressed RORγt functioned properly in mediating T cell development. Interestingly, forced expression of Bcl-xL was also able to restore thymocyte differentiation to a significant level, although not as well as RORγt itself (Fig. 4B, third panel), indicating that one of the major reasons that exogenously introduced RORγt was able to rescue RORγt-deficient thymocyte development was related to enhancement of DP thymocyte survival. Next, we investigated whether forced expression of RORγt was able to rescue TCF-1−/− DP thymocyte survival by transducing TCF-1−/− thymocytes with either empty (Fig. 4C, left panel) or RORγt-expressing retroviruses (Fig. 4C, right panel). Similar to RORγt−/− thymocytes, TCF-1−/− DN thymocytes failed to differentiate into DP thymocytes in the presence of empty viruses. However, retroviral expression of RORγt that had rescued RORγt−/− thymocyte development (Fig. 4B) was clearly able to restore TCF-1−/− thymocyte development (Fig. 4C, right panel). Both the frequency and absolute cell numbers of TCF-1−/− DP thymocytes that expressed exogenous RORγt were significantly increased compared with the cells transduced with empty viruses (Fig. 4C, 4D). To determine the apoptosis status of those differentiated TCF-1 DP thymocytes in the presence of RORγt, we managed to sort out the TCF-1−/− DP thymocytes and assessed the expression of Bcl-xL. As Fig. 4E shows, forced expression of RORγt in TCF-1−/− cells increased Bcl-xL 5–7-fold greater than...
FIGURE 4. Exogenous RORγt rescues TCF-1−/− thymocyte development and restores Bcl-xL levels. A, WT DN thymocytes differentiated into DP thymocytes in the IVD system. Sorted WT (CD4−CD8−) DN thymocytes (left panel) were cocultured with an OP9-DL1 stromal cell layer overnight and then either treated with fresh medium (middle panel) or transduced with empty viruses (MIGR) (right panel). Three days later, cocultures were harvested and stained with Thy1.2, 7-AAD, and Abs against CD4 and CD8 and subjected to flow cytometry. The CD4-CD8 contour of WT thymocytes without viral transduction was gated on Thy1.2+7-AAD− and that of thymocytes transduced with viruses was gated on Thy1.2+7-AAD−GFP+. B, Rescue of RORγt−/− DP thymocyte survival by forced expression of Bcl-xL or RORγt. Cocultures of OP9-DL1 cells and RORγt−/− thymocytes that were untreated (far left panel) or were transduced with MIGR (second panel from left), MIGR-Bcl-xL (third panel from left), or MIGR-RORγt (far right panel) viruses were harvested and stained as described in A. Untreated thymocytes were gated on Thy1.2+7-AAD−, and the rest were gates on Thy1.2+7-AAD−GFP+. Data in A and B are the representative of four independent experiments. C, Rescue of TCF-1−/− DP thymocyte development by forced (Figure legend continues)
FIGURE 5. Forced expression of TCF-1 in RORγt−/− thymocytes fails to rescue DP thymocyte development. Cocultures of OP9-DL1 and RORγt−/− thymocytes transduced with MIGR-RORγt (left panel), MIGR-TCF-1 (middle panel), or MIGR-β-catenin (constitutively activated β-catenin) (right panel) were harvested, and flow cytometry was performed as described in Fig. 4. The results are representative of at least three separate experiments.

in the original TCF-1–deficient thymocytes, indicating that the accelerated apoptosis exhibited by TCF-1–deficient thymocyte was prevented by the ectopically expressed RORγt. Thus, our data suggested that the prevention of DP thymocyte apoptosis is at least partially responsible for the rescue of TCF-1–deficient thymocyte development by ectopically expressed RORγt. Taken together, these data strongly supported the genetic order whereby TCF-1 acts upstream of RORγt, which eventually leads to upregulation of Bcl-xL and enhancement of DP thymocyte survival.

Ectopic expression of TCF-1 in RORγt−/− thymocytes fails to rescue thymocyte development

As a control, we also decided to test whether ectopically introduced TCF-1 has any impact on accelerated apoptosis exhibited by RORγt-deficient thymocytes. To demonstrate this, using the IVD system, we transduced RORγt-deficient DN thymocytes with TCF-1 or active β-catenin–expressing retroviruses and assessed the development status of the thymocytes (Fig. 5). As a control, retrovirus expressing RORγt rescued DP cells (Fig. 5, left panel). In contrast, viruses that expressed TCF-1 (Fig. 5, middle panel) failed to rescue RORγt-deficient thymocyte development. Neither was the constitutively active β-catenin able to do so (Fig. 5, right panel), consistent with the rapid thymocyte apoptosis that we observed in β-caten−/−/RORγt−/− mice (Fig. 3). Thus, the functional assays both in vitro and in vivo, combined with our microarray data, clearly demonstrated that β-catenin/TCF-1–regulated Bcl-xL expression and DP thymocyte survival are mediated by RORγt.

β-catenin/TCF-1 targets and stimulates RORγt promoter

Lastly, we investigated how β-catenin/TCF-1 regulates RORγt expression in thymocytes. We first determined whether β-catenin/TCF-1 regulates RORγt promoter activity. A RORγt luciferase reporter was constructed to contain 1-kb DNA fragment upstream of the RORγt translation starting site (Fig. 6A). A TCF-1 reporter (TOPFlash) was initially used to test the expression plasmids for β-catenin, TCF-1, and dominant-negative TCF (dnTCF) that can block WT TCF-1 activity (18) (Fig. 6B). Indeed, TCF-1 reporter was stimulated by WT and more potently by the constitutively active β-catenin. The greatest reporter activity was observed in the presence of both WT TCF-1 and active β-catenin, whereas dnTCF-1 prevented active β-catenin from stimulating the TCF-1 reporter, indicating the expression plasmids for WT and active β-catenin, as well as WT TCF-1 and dnTCF, functioned as expected. Similar to the stimulation of TOPFlash, ectopically expressed β-catenin, the stabilized β-catenin also led to a significant activation of the RORγt reporter (Fig. 6C). Furthermore, WT TCF-1 potentiated, whereas dnTCF inhibited, β-catenin–mediated activation of the RORγt reporter, suggesting that β-catenin/TCF-1 is able to stimulate RORγt gene expression by activating its promoter activity. Next, we performed ChIP assays to examine whether β-catenin directly interacts with the RORγt promoter region in thymocytes (Fig. 6D). As a positive control, a fragment of Runx2 promoter (−137 to +22 bp), which was previously shown to be occupied by β-catenin/TCF-1 in mouse osteoprogenitor MC3T3 cells (21), was enriched by the β-catenin–Ab-precipitated chromatin from both WT and β-catenin β-caten−/− thymocytes. To determine whether there is any enrichment for RORγt promoter region, we designed three primer sets, each of which flanks the three putative TCF-1–binding motifs (CCTTTG) (RORγt+47, RORγt−302, and RORγt−680) in the RORγt promoter region (Fig. 6A), and found that the 200-bp fragment encompassing the CTTTG motif at +47 (RORγt+47) was enriched by 7- and 90-fold in WT (Fig. 6D, left panel) and β-catenin–transgenic (Fig. 6D, right panel) thymocytes, respectively. In addition, the fragments harboring each of the other two CTTTG motifs (RORγt−302 and RORγt−680) were enriched, although at a much lower level. In contrast, a fragment located at −4.5-kb upstream of RORγt translation starting site was not detectable in the enriched chromatin (negative control). The fact that much greater enrichment was detected in β-caten−/− thymocytes, which also has significantly higher RORγt levels than WT cells, strongly suggested that β-catenin/TCF-1 complex specifically binds to RORγt regulatory region and stimulates RORγt transcription. To demonstrate the function of the putative TCF-1 responsive element(s) on the RORγt promoter activity, we mutated each of the three putative TCF-1 motifs from CTTTG to CCTCG (Fig. 6E, left panel), which abolished the capability of the motif to mediate the binding of TCF-1 to Runx2 promoter (21), and evaluated the expression of RORγt. Coculture of OP9-DL1 cells and TCF-1−/− thymocytes transduced with MIGR (left panel) or MIGR-RORγt viruses (right panel) were harvested, and flow cytometry was performed as described in A. All thymocytes were gated on Thy1.2+7-AAD− GFP+ cells. The absolute numbers of DN, DP, CD4+ SP, and CD8+ SP thymocytes harvested from C, E. Forced expression of RORγt restores Bcl-xL levels in TCF-1−/− thymocytes. CD4+CD8+ GFP+ thymocytes differentiated from C were electronically sorted, RNA was extracted, and cDNA was subjected to qRT-PCR to measure the RNA levels of RORγt (left panel) and Bcl-xL (right panel). The amplification was normalized to GAPDH mRNA expression. Results in C–E are representative of three separate experiments, and the data in E are mean ± SD (n = 2).
FIGURE 6. β-catenin/TCF-1 targets and stimulates promoter of RORγt. A, A schematic diagram depicting a 1-kb RORγt promoter region located immediately upstream of the translation starting site. The three putative TCF-1–responsive elements (CTTTG) are shown in gray squares. The three primer sets used in the ChIP assay are indicated by pairs of arrows (→). “+1” marks RORγt transcription starting site, and “ATG” stands for the translation start site. The structure of the reporter construct used to monitor the activity of RORγt promoter is shown at the top. B, β-catenin, together with WT TCF-1, but not dnTCF, stimulates TOPFlash reporter activity. 293T cells were cotransfected with TOPFlash reporter and the expression vectors for β-catenin (WT or stabilized β-catenin [act β-catenin]), TCF-1, or β-catenin together with TCF-1 (WT or dnTCF-1), and luciferase activity was measured and normalized to Renilla luciferase activity. C, Significant stimulation of the activity of a RORγt reporter by β-catenin/TCF-1. The reporter harboring the 1-kb RORγt promoter region was transfected into Jurkat cells together with empty vector, WT or stabilized β-catenin (act β-catenin), TCF-1, or the combination of β-catenin and TCF-1 (WT or dnTCF-1) expression vectors. “Folds of stimulation” was obtained as in B. D, The RORγt promoter fragment containing the putative TCF-1 binding sites was enriched by anti–β-catenin Ab. Thymocytes from WT (left panel) or β-catenin Tg (right panel) mice were isolated and lysed, and ChIP assay was carried out using Abs against β-catenin. Precipitated DNA was extracted, and the enrichment for specific RORγt promoter fragments was quantified by qRT-PCR using primers targeting the three fragments, each of which contains a putative TCF-1 binding site (RORγt+47, RORγt−2302, and RORγt−2680). The enrichment for Runx2 promoter (−2137 to +22) (“Runx2”) and a fragment 4.5 kb upstream of RORγt translation starting site (“negative control”) were used as positive and negative controls, respectively. “Relative enrichment” was obtained by Ct of Ab-treated groups minus IgG-treated groups (ΔCt), normalized by input difference. E, Destruction of TCF-1 binding sites led to a significant reduction in RORγt promoter activity. Each (RORγt+47M, RORγt−302M, and RORγt−680M) or all three (RORγt-M) “CTTTG” motifs located within the RORγt promoter region were mutated to “CCTCG,” and the activity of mutated RORγt promoters was measured by reporter assays in the presence of ectopically expressed constitutively active β-catenin. The mutagenesis of CTTTG motif (left panel), with WT (CTTTG) and mutated (CCTCG) motifs represented by solid and hatched bars, respectively. Reporter assay results (right panel). All results are presented as mean ± SD (n = 2) and represent at least three separate experiments.
activity of the mutated ROR\textgamma promoter (ROR\textgamma+47M, ROR\textgamma−305M, and ROR\textgamma−680M) by luciferase reporter assays (Fig. 6E, right panel). Consistent with the maximal levels for enrichment for the fragment encompassing CTTF at +47, there was a 50\% reduction in ROR\textgamma promoter activity when the motif at +47 was mutated (ROR\textgamma+47M) compared with the reporter harboring the WT ROR\textgamma promoter. Although the individual destruction of the other two potential TCF-1 binding sites (ROR\textgamma−305M and ROR\textgamma−680M) did not result in obvious change in the ROR\textgamma promoter activity, destruction of all three TCF-1 sites (ROR\textgamma−305M) resulted in further reduction of ROR\textgamma reporter activity. These results suggested that TCF-1 binding motif located at +47 is the major \beta-catenin/TCF-1–responsive element; however, all three TCF-1 are required to work cooperatively to maximally stimulate ROR\textgamma promoter activity.

**Discussion**

Previous studies, including ours, demonstrated that TCF-1 and ROR\textgamma are important molecules required to support DP thymocyte survival by upregulating the antiapoptotic factor Bcl-x\textsubscript{l} (4, 6, 10, 13). However, the relationship between the TCF-1 and ROR\textgamma pathways during regulation of thymocyte survival was not clear. There are several possibilities, including that TCF-1 and ROR\textgamma work via independent pathways, ROR\textgamma is upstream of TCF-1, or TCF-1 is upstream of ROR\textgamma. We demonstrated that the TCF-1 activator, \beta-catenin, failed to enhance thymocyte survival in the absence of ROR\textgamma and that forced expression of ROR\textgamma rescued TCF-1−/− thymocyte development by upregulating expression of Bcl-x\textsubscript{l}. Thus, both gain- and loss-of-function analyses strongly suggested a genetic link between TCF-1 and ROR\textgamma in the regulation of thymocyte survival and excluded the possibility that the two work through independent pathways. This result also placed the TCF-1 pathway upstream of ROR\textgamma, which was further supported by the upregulation of ROR\textgamma in \beta-catenin−/− mice and downregulation of ROR\textgamma in TCF-1−/− mice. Therefore, our studies demonstrated a genetic axis “\beta-catenin/TCF-1→ROR\textgamma→Bcl-x\textsubscript{l},” in the Bcl-x\textsubscript{l}−mediated DP thymocyte survival-regulatory pathway.

The earliest T cells in the thymus are CD4\textsuperscript{+}CD8\textsuperscript{−} DN thymocytes. Survival of early DN cells depends on IL-7, until a TCR\beta chain is successfully rearranged and assembled with pre-TCR\alpha to form pre-TCR. DN cells that express a functional pre-TCR signals required for thymocyte development; therefore, it is believed that TCF-1 may be part of the pre-TCR activation signals. This raises the possibility that pre-TCR signals may participate in activation of the TCF-1 pathway, which remains to be tested. Together, these results favor the idea that activated TCF-1, by Wnt\textsubscript{ROR}\textgamma, could bind and activate TCF in certain experimental systems (29). Therefore, we cannot exclude the possibility that TCF-1 is activated by other pathways in thymocytes. As an additional complication, Wnt signals can also be mediated by \beta-catenin–independent noncanonical pathway (30), the function of which in thymocytes is mostly unknown.

Another intriguing question is what signals activate \beta-catenin/ TCF-1. In the canonical pathway, \beta-catenin is activated by Wnt via surface Frizzled receptors (31), and a role for Wnt in T cell development has been indicated (32, 33). Pongracz et al. (34) showed that thymic stromal cells preferentially express Wnt, whereas thymocytes express Frizzled receptors, suggesting the thymic stroma provides Wnt signals to thymocytes. In agreement with this result, we showed that thymocytes downregulate \beta-catenin after separation from the thymic stroma (6). Because there are multiple isoforms of Wnt, it remains to be determined which Wnts are responsible for activation of \beta-catenin in thymocytes. In addition, it is unlikely that \beta-catenin/TCF-1 is the only pathway required to turn on ROR\textgamma expression, because pre-TCR may also play a role in the upregulation of ROR\textgamma (35). This then raises the question of the relationship between TCF-1 and pre-TCR signals in the induction of ROR\textgamma. Gounari et al. (36) showed that activation of the TCF-1 pathway could partially replace pre-TCR signals required for thymocyte development; therefore, it is believed that TCF-1 may be part of the pre-TCR activation signals.

In addition to TCF-1 and ROR\textgamma, other signaling molecules were reported to regulate thymocyte survival. In contrast to TCF-1, Egr3 was shown to negatively regulate thymocyte survival, presumably by inhibiting ROR\textgamma (37). An interesting model that remains to be tested is whether Egr3 prevents TCF-1 from stimulating ROR\textgamma expression. Mice lacking NFAT4, which is preferentially expressed in DP thymocytes, displayed significantly increased DP thymocyte apoptosis (38). Interestingly, GSK-3\textbeta, which induces degradation of \beta-catenin by phosphorylation, is also a critical regulator for NFAT nuclear localization (39). Therefore, it is likely that GSK-3\textbeta is a critical molecule for cross-talk between these two pathways that regulate thymocyte survival. It remains to be determined whether NFAT4-regulated survival is related to ROR\textgamma. Considering the critical function of apoptosis in thymocyte development, it is not surprising that multiple pathways have been implicated in thymocyte apoptosis. Most of the pathways described above that either positively or negatively regulate thymocyte survival are linked to ROR\textgamma-mediated upregulation of Bcl-x\textsubscript{l}; therefore, it appears that ROR\textgamma is a critical joining point shared by these survival pathways, as demonstrated in this study for \beta-catenin/TCF-1–regulated survival.
Acknowledgments

We thank Dr. Timothy Bender (University of Virginia), who offered great help in the establishment of the in vitro thymocyte differentiation system. We are also thankful to Dr. John Shively and Dr. Ellen Rothenberg for inspiring discussions and suggestions, Dr. Takahiro Maeda for kindly offering Phoenix cells, and Dr. Yunfei Liang for assistance with the flow cytometry.

Disclosures

The authors have no financial conflicts of interest.

References