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Down-Regulation of the Orphan Nuclear Receptor RORγt Is Essential for T Lymphocyte Maturation

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Thymocyte development is a tightly regulated process. CD4⁺CD8⁺ double-positive (DP) immature thymocytes exhibit distinct phenotypic features from mature T cells; they express only 10% of surface TCR that are found on mature T cells and do not proliferate and produce IL-2 in response to stimulation. In this report, we show that transgenic expression of the orphan nuclear receptor RORγt in mature T cells down-regulates their surface TCR expression. The RORγt transgene inhibits IL-2 production by mature T cells, and this inhibition may be partially due to the inhibitory effect of RORγt on c-Rel transcription. Furthermore, ectopic expression of RORγt inhibits the proliferation of mature and immature T cells. These results, together with its predominant expression in DP thymocytes, suggest that RORγt controls these distinct phenotypic features of DP thymocytes. Our data suggest that down-regulation of RORγt expression in thymocytes is essential for their maturation. The Journal of Immunology, 2000, 164: 5668–5674.
Materials and Methods

Cell lines

The KMI-8.3.5 cell line (20) is a T cell hybridoma. KMI-8.3.5ROR
and its control cell line expressing hCD2 were described previously (16). 
KMI-8.3.5ROR expressing c-Rel was generated by retroviral transduc-
tion of a full-length c-Rel cDNA using the pM15 vector, which is identical 
with the pM1 vector (16) except that it contains an internal ribosomal entry 
site (IRES)-driven hCD5, followed by multiple rounds of panning on anti-
hCD5 mAb-coated plates. Cells were cultured in DMEM containing 10% 
FCS, 2 mM glutamine, 25 mM HEPES, 50 mM 2-ME, 100 U/ml penicillin, 
and 100 µg/ml streptomycin.

Abs and reagents

Polyclonal rabbit anti-mouse RORγt serum was generated against the C-
terminal 12-aa peptide of RORγt and affinity-purified using SulfoLink 
kit (Pierce, Rockford, IL) according to the manufacturer’s instructions. The 
following mAbs were purchased from PharMingen (San Diego, CA): pu-
rified and FITC- or Cy-Chrome-anti-CD3 (145-2C11); FITC-, PE- or Cy-
Chromo-anti-CD4 (H129.19); PE- or Cy-Chrome-anti-CD8α (53-6.7); 
FITC-anti-CD25 (7D4); PE-anti-CD44 (1M7); PE-anti-CD69 (H1.2F3); 
biotin-anti-FasL (MFL3); biotin-anti-B20 (RA3-6B2); biotin-anti-Mac-1 
(M1/70); FITC-anti-hCD2 (RPA-2.10); FITC-anti-hCD5 (UCHT2); and 
biotin-anti-I-Aβ (KH74). Rabbit polyclonal anti-c-Rel Ab was purchased 
from Santa Cruz Biotechnology (Santa Cruz, CA). PMA and ionomycin 
were purchased from Calbiochem (La Jolla, CA). Recombinant human 
IL-2 was obtained from Chiron (Emeryville, CA).

Mice

The RORγt transgenic construct was generated by inserting a cDNA frag-
ment encoding the full-length RORγt into the EcoRI site of the VαhCD2 
transgenic vector (21). The resultant construct was released with XhoI/Xbal 
and injected into C57BL/6 x DBA2/F1 embryos. Founder mice were 
identified by Southern blot analysis of tail DNA and backcrossed to 
C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME). All mice were 
housed under specific pathogen-free conditions in the animal facility of 
the University of Washington.

Northern blot analysis and RT-PCR

Total RNA was extracted from cell lines using STAT-60 (Tel-Test, 
Friendswood, TX) and analyzed by Northern blot analysis using a standard 
protocol (22). The cDNA probe for c-Rel was derived by RT-PCR. RT-
PCR for RORγt mRNA expression was described previously (16). The 
PCR products were analyzed on 1% agarose gel.

Western blot analysis

Hybridoma cells were lysed in lysis buffer (10 mM HEPES, 40 mM KCl, 
3 mM MgCl2, 1 mM DTT, 5% glycerol, 0.2% Nonidet P-40, 1 µg/ml 
aprotinin, 1 µg/ml leupeptin, and 1 mM PMSF) on ice for 10 min, and the 
isoluble portion was removed by centrifugation. Primary cells were 
lysed with SDS sample buffer at 100°C for 10 min. Equal number of cells (0.5– 
10⁶ cells/lane) were run on 8 or 10% polyacrylamide gels and trans-
ferred to nitrocellulose. The membranes were then probed with Abs to 
mRORγt, c-Rel, followed by HRP-conjugated secondary Abs and de-
tected with enhanced chemiluminescence according to the manufacturer’s 
instructions (Amersham, Arlington Heights, IL).

Cell separation and flow cytometric analyses

TN subsets and CD4⁺ or CD8⁺ SP thymocytes were first enriched by 
negative selection with biotin-labeled Abs and streptavidin-labeled Dyna-
beads (Dynal, Oslo, Norway) followed by FACS sorting. The purity of 
sorted subpopulations of thymocytes was >99% in postsort analyses. Pe-
niperal T cells from spleen or lymph nodes were purified by negative 
selection with biotin-labeled anti-B220, anti-Mac-1, and anti-I-Aβ, fol-
lowed by incubation with streptavidin-labeled Dynabeads. The purity of 
these cells was >90%. Cells were sequentially incubated with an excess of 
biotinylated mAb, PE-streptavidin, and FITC- or Cy-Chrome-labeled Abs 
on ice and washed with PBS containing 0.1% BSA. Data were collected for 
5 × 10⁶ cells on a FACScan flow cytometer (Becton Dickinson, Mountain 
View, CA) using CellQuest software.

Cell proliferation assay and IL-2 production

Purified T cells (2–5 × 10⁹/well) were added to 96-well tissue culture 
plates in the presence of PMA (10 ng/ml) plus ionomycin (0.1 µg/ml) for 
the indicated time. The supernatants were removed for IL-2 assay. IL-2 
was measured using an ELISA kit (PharMingen, San Diego, CA) according to 
the manufacturer’s instructions. In the proliferation assays, cells were 
labeled with [3H]thymidine (1 µCi/well, 25 Ci/mmol; New England Nu-
clear, Boston, MA) for 4 h, harvested on glass-fiber filters, and counted in 
a beta scintillation counter. Data were derived from the mean of duplicate 
or triplicate cultures, with an SD < 10%.

Results

Expression of RORγt in thymocyte subpopulations

We previously demonstrated that the expression of RORγt in T cells 
is developmentally regulated (16). RORγt mRNA was detected at a 
high level in DP thymocytes and at a low level in CD4⁺ CD8⁻ (DN) 
thymocytes, and was undetectable in CD4⁺ or CD8⁺ SP thymocytes or 
splenic T cells (16). To establish more precisely the expression pattern 
of RORγt during thymocyte maturation, we performed RT-
PCR analysis of FACS-sorted immature DP, intermediate 
CD4⁺ CD8⁻ HSAhigh, and mature CD4⁺ CD8⁻ HSAlow thymocytes 
(23, 24). As shown in Fig. 1A, expression of RORγt mRNA is 
versely correlated with the maturity of thymocytes. Immature DP thy-
mocytes express the highest level of RORγt mRNA, while interme-
iate CD4⁺ CD8⁻ HSAhigh thymocytes on the pathway to SP stage 
express lower levels of RORγt. Fully mature CD4⁺ CD8⁻ HSAlow 
thymocytes do not express RORγt. These results demonstrate that thym-
ocytes gradually down-regulate RORγt expression as they undergo 
maturaton from the DP to the SP stage. We also examined the expression of RORγt within the 
CD3⁺ CD4⁺ CD8⁻ (TN) thymocyte subset. TN thymocytes can be 
further divided into four subsets of progressive maturity based on 
the expression of CD44 and CD25 (25). The earliest cells are 
CD4⁺ CD25⁻ (stage 1), followed by CD4⁺ CD25⁺ (stage 2), 
CD4⁺ CD8⁻ (stage 3), and CD4⁺ CD25⁺ (stage 4). TN thy-
mocytes at different stages were purified by FACS sorting and 
analyzed for RORγt expression by RT-PCR. Among the four 
sets of TN thymocytes, RORγt mRNA was detected at a high 
level only in cells from stage 1 (Fig. 1B). In contrast, the expres-
sion of RORγt mRNA was very low or undetectable in TN thy-
mocytes from stage 2 through stage 4 (Fig. 1B), indicating a tightly 
controlled expression of RORγt within the TN thymocyte com-
partent. These results correlate with our previous observation of 
a low level of RORγt mRNA in unseparated DN thymocytes (16)

Generation of transgenic mice expressing RORγt

Given the tightly controlled expression pattern of RORγt in T 
lymphocytes, we reasoned that ectopic expression of RORγt in mature 
T cells may provide insight to its function. To achieve this, we 
generated transgenic mice using the hCD2 promoter to drive 
RORγt expression in immature DN, DP, and mature T cells. The 
VahCD2 transgenic vector specifically directs transgene expres-
sion in all T cells in a copy number-dependent fashion (21). Two 
independent RORγt transgenic founder lines (founders 850 and 
779) with ~10 and 20 copies, respectively, of transgene were es-
tablished. Progeny from these two lines exhibited a similar phe-
notype, and herein results from one line (founder 779) are re-
ported. The expression of RORγt mRNA was readily detected in 
CD4⁺ or CD8⁺ SP cells purified from the thymus and spleen or 
lymph nodes of RORγt transgenic mice, but not in cells from 
littermate controls (Fig. 1C). RORγt transgene expression was fur-
ther confirmed by Western blotting. In agreement with the RT-
PCR data, RORγt protein was expressed in purified T cells from 
the spleen or lymph nodes of transgenic mice but not from litters

mate controls (Fig. 1C). Importantly, the level of RORγt protein 
expression in peripheral T cells from transgenic mice was ~5-fold 
lower compared with the expression level of RORγt in thymocytes 
from control mice (Fig. 1D). Despite this low level of protein 
expression in mature T cells, RORγt transgene had a clear effect
on their phenotype and function (see below). These results indicate that the hCD2 transgenic vector targeted ROR\textsuperscript{gt} to mature T cells and are consistent with its capacity to target gene expression in all T cells.

The ROR\textsuperscript{gt} transgene blocks T cell development at an early stage

T cell development was examined in ROR\textsuperscript{gt} transgenic mice. Compared with littermate controls, the thymic cellularity of ROR\textsuperscript{gt} transgenic mice was severely reduced (Fig. 2A). On the average, the number of thymocytes from ROR\textsuperscript{gt} mice was reduced 85%. Total cell numbers from spleen and lymph nodes of ROR\textsuperscript{gt} mice were also reduced 50–60% compared with those from the littermate controls (Fig. 2A). T cell development was further characterized by FACS analysis using CD4 and CD8 as surface markers. ROR\textsuperscript{gt} transgenic mice had a dramatically lower percentage of DP thymocytes and a higher percentage of DN thymocytes compared with control mice (Fig. 2B). Although the percentage of CD4\textsuperscript{+} SP or CD8\textsuperscript{+} SP thymocytes in ROR\textsuperscript{gt} mice was relatively higher, the absolute cell number of each of these two subsets was still lower than that in control mice (Fig. 2). The CD8 surface level of the Figure 2A and B.
on DP thymocytes from the transgenic mice was relatively lower than that on control cells (Fig. 2A). Interestingly, a significant number of thymocytes in RORγt mice were either CD4lowCD8− or CD4+ CD8low (Fig. 2B). These cells did not appear to be an abnormal expansion of some minor populations within the thymus, because they did not express c-Kit, NK1.1, CD25, or TCRβδ (not shown). Furthermore, these cells were not present in the spleen of the RORγt transgenic mice (Fig. 2B). The CD4/CD8 profile of splenocytes from the RORγt transgenic mice was relatively normal, except for a lower percentage of CD8+ T cells (Fig. 2B). These data suggest that the expression of RORγt transgene blocked thymocyte development, possibly at the transition from the DN to the DP stage.

To define the developmental stage that was blocked by the RORγt transgene, we analyzed T cell precursors within the TN thymocyte compartment. In contrast to control TN thymocytes, RORγt mice exhibited an altered pattern of distribution of subsets defined by the CD44 and CD25 markers (Fig. 2B). A large fraction of the TN thymocytes from transgenic mice accumulated as CD44+CD25− (stage 3) cells, whereas few CD44+CD25+ (stage 4) cells were detected in RORγt mice (Fig. 2B). The lower expression of CD44 on stage 1 TN thymocytes of transgenic mice was not consistent among different mice (Fig. 2B). To test whether the reduced thymocyte cellularity is due to an enhanced apoptosis, we examined all the subsets of thymocytes defined by expression of CD4 and CD8 or CD25 and CD44 in combination with annexin V. No difference was found in the frequency of annexin V+ apoptotic thymocytes between control and RORγt mice (not shown). Taken together, these results indicate that the expression of the RORγt transgene blocked thymocyte development within the TN compartment, possibly by inhibiting the proliferative expansion of CD25+CD44+ thymocytes (stage 4) or the differentiation of the CD25−CD44− subset (stage 3) to stage 4.

**RORγt down-regulates TCR expression**

We next examined the effect of the RORγt transgene on the surface expression of TCR by mature T cells. Surprisingly, both CD4+ and CD8+ SP thymocytes from RORγt mice expressed lower levels of TCR on their surface (Fig. 3A). The level of TCR expression on RORγt transgenic SP thymocytes is about half that of control thymocytes as assessed by both anti-CD3 (Fig. 3A) and anti-TCRαβ (not shown) mAb staining. Correlating to its predominant expression of endogenous RORγt in DP thymocytes, the low level of TCR expression in DP thymocytes was not further reduced by ectopic RORγt expression (Fig. 3A). In addition, ~30% of the CD4+ SP thymocytes from RORγt mice expressed low or undetectable levels of CD3 on their surface (Fig. 3A). A similar decrease in the TCR level was found in peripheral T cells from the spleen (Fig. 3B) and lymph nodes (not shown) of RORγt mice. Furthermore, ectopic expression of RORγt in KMs-8.3.5 hybridoma cells decreased TCR surface expression (not shown). These results demonstrate that ectopic expression of RORγt in mature T cells down-regulates TCR surface expression.

**RORγt inhibits IL-2 production**

RORγt was shown to inhibit IL-2 production by a hybridoma cell line (16). To investigate whether ectopic expression of RORγt in mature T cells inhibits their ability to produce IL-2, we purified CD4+ SP thymocytes or splenic T cells from RORγt mice or littermate controls and stimulated these cells with PMA plus ionomycin, which activate T cells by bypassing TCR. Both CD4+ SP thymocytes and splenic T cells from RORγt transgenic mice produced dramatically lower amounts of IL-2 compared with cells from littermate controls (Fig. 4A). Therefore, ectopic expression of RORγt in mature T cells inhibited their ability to produce IL-2.

**RORγt negatively regulates c-Rel expression**

IL-2 expression is controlled by multiple transcription factors (11). T cells from c-Rel-deficient mice have impaired IL-2 production (26, 27). To test whether the inhibition of IL-2 production by RORγt is due to an effect on c-Rel expression, we performed Northern blot analyses to determine the effect of RORγt on c-Rel induction in the T cell hybridoma KMs-8.3.5. Following activation, the expression of c-Rel mRNA was induced as early as 1 h and continuously increased up to 6 h in control KMs-8.3.5 hCD2 cells (Fig. 4B). In contrast, the induction of c-Rel mRNA was strongly inhibited in KMs-8.3.5RORγt cells (Fig. 4B). This result was confirmed by Western blot analysis (Fig. 4C). Although we did not detect c-Rel mRNA in unstimulated KMs-8.3.5 cells (Fig. 4B), these cells express a detectable level of c-Rel protein before activation and dramatically up-regulate its expression after activation (Fig. 4C). In contrast, KMs-8.3.5RORγt cell lines expressed significantly less c-Rel protein both before and after activation by PMA plus ionomycin (Fig. 4C). Importantly, the expression of c-Rel in RORγt transgenic T cells was also reduced (Fig. 4D). These results demonstrate that RORγt negatively regulates c-Rel transcription.

To test whether the inhibition of IL-2 production by RORγt is solely due to its inhibition of c-Rel transcription, we transduced a full-length c-Rel cDNA into KMs-8.3.5RORγt using a retroviral vector containing an IRES-hCD5 reporter cassette. Constitutive expression of c-Rel in this cell line did not restore its capacity to produce IL-2 (not shown), suggesting that RORγt may additionally regulate other genes that are required for IL-2 production.
RORγt inhibits T cell proliferation

To examine the effect of ectopic expression of RORγt on T cell proliferation, we stimulated purified CD4⁺ SP thymocytes or splenic T cells with PMA plus ionomycin. As shown in Fig. 5A, the proliferation of mature T cells from both the thymus and spleen was significantly inhibited by the RORγt transgene. T cells from c-Rel-deficient mice exhibited a defect in proliferation, and this defect can be corrected by adding exogenous IL-2 (26, 27). When exogenous IL-2 was added to the cell culture, the proliferation of the T cells from the RORγt transgenic mice was slightly increased but still significantly lower than that in control T cells (Fig. 5A). To determine whether the decreased proliferation of the transgenic T cells is due to an effect on their ability to be activated, we analyzed these cells for the expression of the T cell activation markers CD69 and CD25. After 48 h of activation, splenic T cells from RORγt transgenic mice exhibited a defect in proliferation, and this defect can be corrected by adding exogenous IL-2. T cells from c-Rel-deficient mice exhibited a defect in proliferation, and this defect can be corrected by adding exogenous IL-2 (26, 27). When

FIGURE 4. Expression of RORγt transgene inhibits IL-2 production and c-Rel up-regulation. A. Expression of RORγt transgene inhibits IL-2 production by mature T cells. FACS-sorted CD4⁺ SP thymocytes or purified splenic T cells in 96-well plates were activated with PMA plus ionomycin for 24 or 48 h, respectively. The supernatants were assayed for IL-2 using an ELISA kit. Data are representative of two experiments. B. Northern blot analysis of c-Rel mRNA expression in KMs-8.3.5 hybridoma cells expressing RORγt. Cells were activated in 2C11-coated plates for the indicated time and harvested, and total RNA was extracted. GAPDH was probed as a loading control. C. Western blot analysis of c-Rel protein expression in KMs-8.3.5 hCD2 and KMs-8.3.5 RORγt cell lines. Cells were activated with PMA (10 ng/ml) plus ionomycin (0.4 μg/ml) for 4.5 h and lysed. An equal number of cell equivalents was run in each lane. Shown are two independent KMs-8.3.5 RORγt cell lines. D. Western blot analysis of c-Rel protein expression in purified splenic T cells from RORγt transgenic or control mice. Purified cells were activated with PMA plus ionomycin for 7 h and lysed in SDS sample buffer.

FIGURE 5. Expression of RORγt transgene inhibits T cell proliferation. A. Proliferation of mature T cells from RORγt transgenic mice and littermate controls. FACS-sorted CD4⁺ SP thymocytes or purified splenic T cells were stimulated with PMA plus ionomycin for 48 h and pulsed with [³H]thymidine for an additional 4 h. IL-2 was added at 50 U/ml. Data are representative of three experiments. B. FACS analysis of the up-regulation of CD69 and CD25 on activated splenic T cells from RORγt transgenic mice and littermate controls. Cells were activated with PMA plus ionomycin as described in A for 24 h and double-stained with anti-CD3 plus anti-CD69 or anti-CD25. CD3⁺ cells were analyzed for their expression of CD25 and CD69. The filled histogram profiles at the left represent the staining of resting splenic T cells. C. Cell cycle analysis of CD44⁺CD25⁻ TN thymocytes. CD44⁺CD25⁻ TN thymocytes were FACS sorted, fixed, and stained with propidium iodide. The percentage of cells in each gated region is indicated.
The severely reduced thymic cellularity and the inhibition of T cell proliferation by mature T cells in ROR\(\gamma\) transgenic mice suggest that ROR\(\gamma\) inhibits the proliferative expansion of CD44\(^+\)CD25\(^-\) TN thymocytes. This thymocyte subset proliferates vigorously in a normal thymus (28, 29), and these cells are the immediate precursors of DP thymocytes (25). To directly test the effect of ROR\(\gamma\) transgene expression on the proliferation of this subset, we FACS sorted CD44\(^+\)CD25\(^-\) TN thymocytes and performed cell cycle analysis on these sorted cells. As expected, a large fraction (30%) of CD44\(^+\)CD25\(^-\) TN thymocytes from control mice were in S/G\(_2\)/M phases of the cell cycle (Fig. 5C). In contrast, very few CD44\(^+\)CD25\(^-\) TN thymocytes (3%) from ROR\(\gamma\) transgenic mice were in S/G\(_2\)/M phases of the cell cycle. The number of subdiploid apoptotic cells in this subset from ROR\(\gamma\) transgenic mice was not increased (Fig. 5C). Collectively, these data suggest that ROR\(\gamma\) inhibits T cell proliferation when ectopically expressed.

The effect of ROR\(\gamma\) transgene expression on Fas ligand up-regulation

ROR\(\gamma\) was shown to inhibit Fas ligand expression in T cell hybridomas (16). To determine whether this effect occurs in vivo, we examined the effect of ROR\(\gamma\) transgene expression on Fas ligand up-regulation in mature T cells. Thymocytes and splenocytes were activated with PMA plus ionomycin and stained with anti-CD4, anti-CD8, and Fas ligand mAbs. Histograms at the far left represent Fas ligand staining of fresh thymocytes and splenocytes. Dotted line, stimulated thymocytes from ROR\(\gamma\) transgenic mice; thick line, stimulated cells from littermate controls.

**FIGURE 6.** Effect of the expression of ROR\(\gamma\) transgene on Fas ligand up-regulation by mature T cells from ROR\(\gamma\) transgenic mice. Total thymocytes and splenocytes were activated with PMA plus ionomycin for 72 h and stained with anti-CD4, anti-CD8, and Fas ligand mAbs. Histograms at the far left represent Fas ligand staining of fresh thymocytes and splenocytes. Dotted line, stimulated thymocytes from ROR\(\gamma\) transgenic mice; thick line, stimulated cells from littermate controls.

The inhibition of IL-2 production by ROR\(\gamma\) may be due to its negative effect on c-Rel transcription and on certain other genes that are required for IL-2 production. The transcriptional control of the IL-2 gene has been thoroughly studied. Multiple elements capable of binding AP-1 complexes, NF-AT, NF-kB/Rel, and Oct-1 have been identified in 5' upstream of the IL-2 transcriptional start site (11). Of these various response elements and transcription factors, c-Rel was unequivocally shown to be required for IL-2 production in mouse knockout studies (26, 27). Our in vivo and in vitro results identified ROR\(\gamma\) as a negative regulator of c-Rel transcription. In further support of this, c-Rel mRNA expression is inversely correlated with ROR\(\gamma\) expression. c-Rel mRNA was not detected in the majority of DP thymocytes, but is expressed in positively selected TCR\(^{\alpha\beta}\)DP thymocytes and mature T cells (31), whereas ROR\(\gamma\) is highly expressed in DP thymocytes and is down-regulated in mature T cells. Besides the effect on c-Rel expression, ROR\(\gamma\) might also affect the expression or activities of other genes that are involved in IL-2 production. Constitutive expression of c-Rel in the hybridoma line expressing ROR\(\gamma\) did not restore its ability to produce IL-2. NF-AT and AP-1 are additional transcription factors that may be negatively regulated by ROR\(\gamma\). Although ROR\(\gamma\) has no obvious effect on the expression and nuclear translocation of NF-ATc in the KM3L-8.3.5 hybridoma cell line (Y.-W. He and M. J. Bevan, unpublished observation), other studies have shown that these transcription factors lack DNA binding activity in DP thymocytes (12-15). This raises the possibility that ROR\(\gamma\) may compete with these factors for binding to the IL-2 promoter.

ROR\(\gamma\) has the capacity to inhibit the proliferation of mature and TN immature T cells when ectopically expressed. Given its high level of expression in DP thymocytes, ROR\(\gamma\) may act as an inhibitor of DP cell proliferation. Several lines of evidence indicate that the inhibition of T cell proliferation by ROR\(\gamma\) is not solely due to its effect on c-Rel transcription. Although T cells from c-Rel-deficient mice have impaired capacity in terms of proliferation, this defect can be corrected by adding exogenous IL-2 (26, 27). In contrast, addition of exogenous IL-2 did not correct the deficiency of ROR\(\gamma\) transgenic T cells in proliferation. Furthermore, expression of ROR\(\gamma\) severely reduced thymic cellularity,
whereas T cell development in c-Rel-deficient mice is normal (26, 27). The reduced thymic cellularity in RORγt transgenic mice is probably due to an impaired proliferative expansion of CD44+CD25+ TN thymocytes. Correlating to their low level expression of endogenous RORγt, a large fraction of the normal CD44+CD25+ TN thymocytes is proliferating (28, 29). The expression of the RORγt transgene in this subset decreased the number of cells in the S/G2/M phases of the cell cycle by 90%. It remains to be determined how RORγt negatively regulates T cell proliferation.

The down-regulation of TCR surface expression by RORγt in mature T cells suggests that RORγt control the low levels of TCR surface expression on normal DP thymocytes. Previous studies demonstrated that DP thymocytes express approximately equal amounts of mRNA for each of the TCR components compared with mature T cells (6), but express on the surface only about 10% of the level found on mature T cells (4, 5). Although the physiological significance of the low levels of TCR surface expression is not clear, it appears that most intrathymic repertoire selection occurs among these DP thymocytes expressing low levels of surface TCR (32). How RORγt regulates surface TCR expression will be of interest for future studies.

The effects of RORγt revealed in this study should help us to understand the physiology of DP thymocytes. The most immature DN thymocytes proliferate and produce IL-2 upon stimulation. When DN thymocytes differentiate into more mature DP cells, they lose the capacity for both proliferation and IL-2 production. Not until these cells become fully mature SP thymocytes do they regain this capacity (10). This stage-specific response correlates well with the expression pattern of RORγt. At the DP stage, thymocytes face positive and negative selection. It is important that neither of these selection events should be accompanied by cell division or cytokine production. We propose that the orphan nuclear receptor RORγt is a critical regulator of the DP thymocyte functions.

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