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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.

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 orphan nuclear receptor RORγt (16) is a thymus-specific isoform of RORγ (17–19). The expression of RORγt is tightly regulated in developing thymocytes. DP thymocytes express high levels of RORγt mRNA, while CD4+ or CD8+ SP mature thymocytes do not express RORγt (16). Ectopic expression of RORγt in T cell hybridoma cell lines inhibits Fas ligand up-regulation and IL-2 production without inhibiting early events of T cell activation such as up-regulation of CD69 (16). However, the role of RORγt in T lymphocyte development is not clear. To address this issue, we generated RORγt transgenic mice using the hCD2 promoter/enhancer/locus control region to drive RORγt expression in both mature and immature T cells. We show that ectopic expression of RORγt in mature T cells down-regulates TCR surface expression. Ectopic expression of RORγt also inhibits the proliferation of mature T cells and TN thymocytes. Furthermore, RORγt expression prevents mature T cells from producing IL-2 and inhibits c-Rel up-regulation. Our results demonstrate a phenotypic similarity between the mature T cells from RORγt transgenic mice and normal immature DP thymocytes. These data together with the predominant expression of RORγt in DP thymocytes support the idea that RORγt is an important regulator of DP thymocyte phenotype and function. Furthermore, our data suggest that down-regulation of RORγt expression is essential for the maturation of DP thymocytes into SP thymocytes.
Materials and Methods

Cell lines

The KMls-8.3.5 cell line (20) is a T cell hybridoma. KMls-8.3.5RORγt and its control cell line expressing hCD2 were described previously (16). KMls-8.3.5RORγt expressing c-Rel was generated by retroviral transduction of a full-length c-Rel cDNA using the pM5 vector, which is identical with the pMI 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 μM HEPES, 50 μM 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): purified and FITC- or Cy-Chrome-anti-CD3 (145-2C11); FITC-, PE- or Cy-Chrome-anti-CD4 (H129.19); PE- or Cy-Chrome-anti-CD8α (53-6.7); FITC-anti-CD25 (7D4); PE-anti-CD44 (1M7); PE-anti-CD69 (H.12F3); biotin-anti-FAcL (MFL3); biotin-anti-B220 (RA3-6B2); biotin-anti-Mac-1 (M1/70); FITC-anti-hCD2 (RPA-2.10); FITC-anti-hCD5 (UCHT2); and biotin-anti-I-Ak (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 fragment encoding the full-length RORγt into the EcoRI site of the VaHCD2 transgenic vector (21). The resultant construct was released with Xhol/XhoI and injected into C57BL/6 × DBA2F1 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

Hydridoma 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 insoluble portion was removed by centrifugation. Primary cells were lysed with SDS sample buffer at 100°C for 10 min. Equal number of cells (0.5–5 × 106 cells/lane) were run on 8 or 10% polyacrylamide gels and transferred to nitrocellulose. The membranes were then probed with Abs to hCD2, c-Rel, followed by 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 × 106/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 Nuclear, 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+CD8lowHSAhigh, and mature CD4+CD8lowHSAlow thymocytes (23, 24). As shown in Fig. 1A, expression of RORγt mRNA is inversely correlated with the maturity of thymocytes. Immature DP thymocytes express the highest level of RORγt mRNA, while intermediate CD4+CD8lowHSAhigh thymocytes on the pathway to SP stage express lower levels of RORγt. Fully mature CD4+CD8low HSAlow cells do not express RORγt. These results demonstrate that thymocytes gradually down-regulate RORγt expression as they undergo maturation 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 CD44+CD25− (stage 1), followed by CD44+CD25+ (stage 2), CD44−CD25+ (stage 3), and CD44−CD25− (stage 4). TN thymocytes at different stages were purified by FACS sorting and analyzed for RORγt expression by RT-PCR. Among the four stages of TN thymocytes, RORγt mRNA was detected at a high level only in cells from stage 1 (Fig. 1B). In contrast, the expression of RORγt mRNA was very low or undetectable in TN thymocytes from stage 2 through stage 4 (Fig. 1B), indicating a tightly controlled expression of RORγt within the TN thymocyte compartment. 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 expression 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 established. Progeny from these two lines exhibited a similar phenotype, and herein results from one line (founder 779) are reported. 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 further 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 littermate 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

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on their phenotype and function (see below). These results indicate that the hCD2 transgenic vector targeted ROR-γt to mature T cells and are consistent with its capacity to target gene expression in all T cells.

The ROR-γt transgene blocks T cell development at an early stage

T cell development was examined in ROR-γt transgenic mice. Compared with littermate controls, the thymic cellularity of ROR-γt transgenic mice was severely reduced (Fig. 2A). On the average, the number of thymocytes from ROR-γt mice was reduced 85%. Total cell numbers from spleen and lymph nodes of ROR-γt 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-γt 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⁺ SP or CD8⁺ SP thymocytes in ROR-γt 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

![FIGURE 1. Expression of ROR-γt in normal thymocyte subpopulations and ROR-γt transgenic mice. A, Expression of ROR-γt in thymocyte subsets at different developmental stages. RT reactions of the three thymocyte subsets from C57BL/6 mice were serially diluted 1/3 and subjected to PCR with primers specific for ROR-γt. HPRT RT-PCR was performed from the same RT samples for cDNA template quantity control. B, Expression of ROR-γt in normal TN thymocyte subsets. RT-PCR analysis of the four TN thymocyte subsets was performed as described in A, except samples were serially diluted 1/10. C, Expression of ROR-γt in ROR-γt transgenic mice. RT reactions of FACS-sorted CD4⁺ SP or CD8⁺ SP thymocytes, total thymocytes, spleen, and lymph nodes from ROR-γt transgenic mice or littermate controls were subjected to PCR as described in A without serial dilution. D, Western blot analysis of ROR-γt expression in normal and ROR-γt transgenic mice. Cell lysates from total thymocytes or purified T cells of spleen or lymph nodes were electrophoresed on a 10% polyacrylamide gel, transferred, and blotted with rabbit anti-mROR-γt Ab.](http://www.jimmunol.org/)

![FIGURE 2. Expression of ROR-γt transgene blocks T cell development. A, Total cell number of lymphoid organs from ROR-γt transgenic mice and littermate controls. Cells from thymus, spleen, and lymph nodes of 7- to 13-wk-old mice were enumerated by trypan blue exclusion. The numbers represent the mean value from six mice in each group. B, FACS analyses of T cell development in the thymus and spleen of ROR-γt mice. Cells were stained with either FITC-anti-CD4 and PE-anti-CD8 or FITC-anti-CD25, PE-anti-CD44, and Cy-Chrome-anti-CD3, -CD4, and -CD8. For the analysis of TN thymocyte, CD3⁺, CD4⁺, and CD8⁺ thymocytes were excluded by electronic gating. The percentage of cells in each gated region is indicated.)
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 CD4^low^CD8^- or CD4^+^CD8^- (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 TCRγδ (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 CD4^+^CD25^- (stage 3) cells, whereas few CD4^+^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 CD4^+^CD25^- (stage 4) or the differentiation of the CD25^-CD44^ low (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 KMcis-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 KMcis-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 KMcis-8.3.5-hCD22 cells (Fig. 4B). In contrast, the induction of c-Rel mRNA was strongly inhibited in KMcis-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 KMcis-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, KMcis-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 KMcis-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.

![FIGURE 3](http://www.jimmunol.org/)  
Expression of the RORγt transgene down-regulates surface TCR expression on mature T cells. Thymocytes and splenocytes were analyzed by three-color staining. Shown are the histogram profiles of CD3 staining for different subsets gated as described in Fig. 2B. The numbers are the mean fluorescence intensity of the CD3^- cells.
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 24 h of activation, splenic T cells from RORγt mice up-regulated both CD69 and CD25 on their surface to similar levels as T cells from control mice (Fig. 5B). These results indicate that the inhibition of T cell proliferation by RORγt is not solely due to its effect on IL-2 production or to an inability of T cells to be activated.

**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 KMIs-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 KMIs-8.3.5 hCD2 and KMIs-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 KMIs-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 RORγt transgenic mice suggest that RORγt 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 RORγt transgene expression on the proliferation of this subset, we FAC**S** 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/G2/M phases of the cell cycle (Fig. 5C). In contrast, very few CD44+CD25− TN thymocytes (3%) from RORγt transgenic mice were in S/G2/M phases of the cell cycle. The number of subdiploid apoptotic cells in this subset from RORγt transgenic mice was not increased (Fig. 5C). Collectively, these data suggest that RORγt inhibits T cell proliferation when ectopically expressed.

The effect of RORγt transgene expression on Fas ligand up-regulation

RORγt 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 RORγt transgene expression on Fas ligand up-regulation in mature T cells. 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 RORγt transgenic mice; thick line, stimulated cells from littermate controls.

![FIGURE 6. Effect of the expression of RORγt transgene on Fas ligand up-regulation by mature T cells from RORγt 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 RORγt transgenic mice; thick line, stimulated cells from littermate controls.](image)

**Discussion**

In this report we present evidence that the orphan nuclear receptor RORγt is an important transcription factor regulating multiple phenotypes of DP thymocytes. DP thymocytes represent a major developmental stage in thymocyte ontogeny. These cells make up the majority of the population in the thymus and face a strict selection process. Unlike the mature SP thymocytes and peripheral T cells, DP thymocytes lack the ability to proliferate and produce IL-2 when stimulated, and they express a much lower level of TCR on their surface. The molecular basis for controlling these phenotypes of DP thymocytes is not clear. We demonstrate here that the expression of endogenous RORγt in T cells is inversely correlated with their maturity. As immature DP thymocytes become mature SP, RORγt expression is down-regulated. This down-regulation of RORγt expression occurs at the transcriptional level. Aberrant expression of RORγt in mature T cells, even at a low level as seen in our transgenic mice, conferred some characteristics of immature DP thymocytes on these cells in terms of their TCR expression, IL-2 production, and proliferative capacity. These results together with its predominant expression in the DP thymocytes suggest RORγt is an important regulator of the DP thymocyte phenotype. Furthermore, our data suggest that down-regulation of RORγt expression is essential for the maturation of DP thymocytes into SP thymocytes and peripheral T cells.

The inhibition of IL-2 production by RORγt 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-κB/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 RORγt as a negative regulator of c-Rel transcription. In further support of this, c-Rel mRNA expression is inversely correlated with RORγt expression. c-Rel mRNA was not detected in the majority of DP thymocytes, but is expressed in positively selected TCRhighDP thymocytes and mature T cells (31), whereas RORγt is highly expressed in DP thymocytes and is down-regulated in mature T cells. Besides the effect on c-Rel expression, RORγt 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 RORγt did not restore its ability to produce IL-2. NF-AT and AP-1 are additional transcription factors that may be negatively regulated by RORγt. Although RORγt has no obvious effect on the expression and nuclear translocation of NF-ATc in the KMIs-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 RORγt may compete with these factors for binding to the IL-2 promoter.

RORγt 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, RORγt may act as an inhibitor of DP cell proliferation. Several lines of evidence indicate that the inhibition of T cell proliferation by RORγt 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 RORγt transgenic T cells in proliferation. Furthermore, expression of RORγt 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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References