J. Microbiol. Biotechnol. (2009), **19**(2), 208–214
doi: 10.4014/jmb.0811.618
First published online 31 December 2008

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Differential Expression of Nuclear Receptors in T Helper Cells

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Received: November 11, 2008 / Accepted: December 14, 2008

Steroid hormones have long been known to have a profound influence on the immune system. Although the functions of the nuclear receptors in the development of T cells are fairly well studied, the differential expression of these receptors in T helper cells is poorly understood. Here, we investigated the differential expression of nuclear receptors and coregulators in Th1 and Th2 cells by genome-wide microarray analysis. The result showed that several nuclear receptors and coregulators are differentially expressed in these cells. The result was confirmed by RT-PCR. The result showed that RXRα is highly expressed in Th2 cells. Overexpression of RXRα in a Jurkat human T cell line induced IL4 but not IFN-γ gene expression, suggesting that RXRα plays a selective role in Th1 and Th2 differentiation. In summary, these results suggest that Th1/Th2 differentiation is influenced by differential regulation of nuclear receptors and coregulators.

Keywords: Nuclear receptor, coregulator, Th1, Th2, differentiation, transcription factor

Nuclear receptors constitute a superfamily of ligand-dependent transcription factors that include receptors for steroid hormones, retinoic acid, and thyroid hormone; and orphan receptors for which the ligands have yet to be identified [18]. Several studies have demonstrated a role for a number of nuclear receptors in the regulation of inflammation. These include the glucocorticoid receptor (GR), estrogen receptor (ER), vitamin D receptor (VDR), retinoic acid receptors (RAR), peroxisome proliferator-activated receptors (PPAR), and several orphan receptors, including members of the retinoid-related orphan receptor (ROR) subfamily [18, 38]. Most members of the nuclear receptor superfamily contain a transcriptional activation domain, DNA binding domain, and ligand domain. Upon ligand binding, nuclear receptors change their conformations, form homodimers, bind to their cognate hormone response elements (HREs), recruit coactivators, and enhance their target gene transcription [18, 23, 36].

Helper T cells (CD4 T cells) play a critical role in coordinating immune responses against a variety of pathogens [1, 3, 8, 22]. Helper T cells are divided into at least two subsets, Th1 and Th2 cells, as well as the recently described Th17 cells [32, 37]. Th1 cells mediate cellular immunity by producing IFN-γ, which activates macrophages to kill ingested intracellular pathogens. Th2 cells mediate humoral immunity by producing IL4, IL5, and IL13, which stimulate the elimination of parasites and the differentiation of B cells to produce antibodies. Besides these beneficial roles, however, Th1 and Th2 cells underlie many human diseases, grouped as autoimmune diseases and allergy, respectively [1, 3, 8, 17]. Th1 differentiation requires the cytokine IL12 and the transcription factors, STAT4, STAT1, and T-bet, whereas Th2 cell differentiation requires the cytokine IL4 and the transcription factors STAT6 and GATA3 [1, 3, 8].

Recent studies have shown crucial roles of several nuclear receptors in T helper cell differentiation. Vitamin A enhanced Th2 development *via* the RXR pathway [16, 25, 31], and disruption of RXRα resulted in alteration of the balance of Th1/Th2 and caused exaggerated Th1 responses and reduced Th2 responses [4, 9, 29, 30]. Depletion of RORγ caused a reduction of Th2 immune response in an animal model of allergic asthma [34]. It has been shown that RORγt is the critical transcription factor for differentiation of Th17 cells [14]. Retinoic acid has been shown to inhibit the differentiation of Th17 cells and induces regulatory T cells reciprocally by reducing RORγt expression [24]. Recent study has shown that RORα also plays an important role in Th17 cell differentiation [39].

These studies strongly suggest that T helper cell differentiation is influenced by nuclear receptors and prompted us to find more nuclear receptors that may play roles in T helper cell differentiation. In this study, we searched for nuclear receptors and coregulators that are differentially expressed in Th1 and Th2 cells, by genome-wide microarray analysis, and we found several Th1/Th2-specific nuclear receptors and coregulators. We confirmed this differentiation-specific expression by reverse

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transcription - polymerase chain reaction (RT-PCR). This study shows that Th1/Th2 differentiation may be influenced by differential actions of nuclear receptors and coregulators.

MATERIALS AND METHODS

In Vitro Differentiation of Th1, Th2, and Th17 Cells

Naïve CD4 T cells were isolated from spleen cells from C57BL/6 mice as previously described [13]. *In vitro* differentiation of Th1 and Th2 cells was carried out as previously described [21]. Briefly, naïve CD4 T cells were stimulated with plate-bound anti-CD3 antibody (10 μg/ml) and soluble anti-CD28 antibody (2 μg/ml) for 2 days in the presence of Th1 or Th2 inducing conditions. For Th1 differentiation, 3.5 ng/ml murine IL12, 10 μg/ml anti-IL4 antibody (11B11), and 20 U/ml IL2 were added. For Th2 differentiation, 1,000 U/ml IL4, 10 μg/ml anti-IFN-γ antibody (XMG1.2), and 20 U/ml IL2 were added. For Th17 differentiation, 1 ng/ml human TGF-β, 50 ng/ml IL6, 10 ng/ml IL1β, 1 ng/ml TNF-α, 10 μg/ml anti-IFN-γ antibody (XMG1.2), and 10 μg/ml anti-IL4 antibody (11B11) were added.

RNA Isolation and Microarray Analysis

After 2 days of stimulation, cells were harvested and RNA was extracted as previously described [27] with minor modifications. The labeling, hybridization, and washing were performed according to the instruction provided by the manufacturer (Agilent Technologies Inc., Santa Clara, CA, U.S.A.). Briefly, 2 μg of total RNA was used for fluorescently labeled cRNA synthesis. For hybridization, 3 μg of Cy3- or Cy5-labeled cRNA from naïve and Th1, or naïve and Th2 cells were combined and hybridized to an Agilent 44K mouse whole genome oligonucleotide microarray. The microarray slides were scanned with a Gene 4000B scanner (Axon Instruments, CA, U.S.A.), and the gene expression profiles were analyzed using GenePix v6.0 software.

RT-PCR

Semiquantitative RT-PCR was used to confirm DNA microarray gene expression data as previously described [26]. Total RNA isolated from 2-day stimulated Th1, Th2, and Th17 cells were converted to cDNA using Superscript II reverse transcriptase (Life Technologies). PCR reactions were carried out using *Taq* polymerse (Takara) using the primers listed in Table 1.

Western Blotting

Cell extracts were resolved on 10% SDS-PAGE and transferred to an PVDF membrane (Bio-rad). The membrane was blocked with 5% skim milk in TBST (incubated 1 h at room temperature). The membrane was probed with a rabbit polyclonal antibody against RXRα (Santa Cruz, sc-553), RORγt (Biolegend, 636201), or goat polyclonal antibody against NCoA3 (Santa Cruz, sc-7126), or mouse monoclonal antibody against β-Actin (Santa Cruz, sc-47778) diluted 1:100 in TBST for overnight at 4°C. Then, a horseradish peroxidase (HRP)-conjugated antibody against rabbit or mouse or goat IgG (Bethyl) diluted 1:2,000 in 5% skim milk TBST was added for 1 h at room temperature. The signal was detected on a X-ray film by ECL reaction.

Cell Transfection

Cell transfection was performed as previously described [20]. Briefly, human T cell lymphoma Jurkat cells were culture in RPMI1640 plus 5% fetal bovine serum with antibiotics. Exponentially growing cells (5×10^6) were transfected with 20 μg of CMV-based expression constructs containing various nuclear receptor and coregulator genes [control CMV (pCMV-SPORT6), mouse RORα (MMM1013-63895), mouse RORγ (MMM1013-7511130), mouse NcoA3 (MMM1013-99622114), human RXRα (MHS1010-98051588); Open Biosystems] by electroporation with a Bio-Rad gene pulser (280 V, 960 μF). Cells were rested for 16 h and then stimulated with 50 ng/ml PMA+1 μM ionomycin for 4 h. Total RNA was isolated and analyzed by RT-PCR as described above.

RESULTS

Searching for Nuclear Receptors and Coregulators by DNA Microarray

To search for nuclear receptors and coregulators that are differentially expressed in Th1 and Th2 cells, we performed

Table 1. Primers used for PCR.

Genes	Sequences (5' → 3')	
mRORα	F: CAATGCCACCTACTCCTGTCC	R: GCCAGGCATTTCTGCAGC
mRORγ	F: GCTGTGGGGTAGATGGGATAGA	R: CAGAGGGGGTCAACAACATTTC
mRXRα	F: AATGGTGGTGTGGGCGTGGTGTC	R: ATGGGCGCAGGGTGGCTAATGAG
mNCoA3	F: ACGTGGCGGCGAGTCATTGT	R: TGGCGAGGCAGAAGGGAGTG
mIL4	F: CATCGGCATTTTGAACGAGGTCA	R: CTTATCGATGAATCCAGGCATCG
mIFN-γ	F: CATTGAAAGCCTAGAAAGTCTG	R: CTCATGAATGCATCCTTTTTCG
mIL17	F: TTTAACTCCCTTGGCGCAAAA	R: TTTCCCTCCGCATTGACAC
mHPRT	F: GTTGGATACAGGCCAGACTTTGTTG	R: GAGGGTAGGCTGGCCTATAGGCT
mRORγ1	F : ATGGACAGGGCCCCACAGAGAC	R : TTTCTGCACTTCTGCATGTAGACT
mRORγt	F : ACCTCCACTGCCAGCTGTGTGCTGTC	R : TTTCTGCACTTCTGCATGTAGACT
mRORγ (both)	F : GGGAGATGTGGGAGCGCTGTGC	R : TCCTTCCTCCAGATCACTTTGACAGCCC
hIFN-γ	F: GCAGAGCCAAATTGTCTCCT	R: ATGCTCTTCGACCTCGAAAC
hIL4	F: CCAACTGCTTCCCCCTCTG	R: TCTGTTACGGTCAACTCGGTG
hIL17	F: TACTACAACCGATCCACCTCACCT	R: CAGCCCACGGACACCAGTATCT
hHPRT	F: GGAGATGTGATGCCGGAGATGG	R: GGATTATACTGCCTGACCAAGG

Table 2. Genes selectively expressed in Th1 cells identified by microarray.

GeneBank Acc. No.	Name	Th1/naïve	Th2/naïve	Th1/Th2
NM_013646	RAR-related orphan receptor alpha	3.477	1.169	2.974
NM_010444	Nuclear receptor subfamily 4, group A, member 1	0.605	0.312	1.939
NM_013613	Nuclear receptor subfamily 4, group A, member 2	10.56	6.991	1.511
AK020076	"*Mus musculus* 13 days embryo male testis cDNA, RIKEN full-length enriched library; clone:6030458L20, product: nuclear receptor interacting protein 1, full insert sequence. [AK020076]"	0.196	0.135	1.452
NM_172622	Transcriptional regulating factor 1	2.494	1.754	1.422
NM_008904	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	0.371	0.263	1.411
NM_020005	P300/CBP-associated factor	0.362	0.271	1.336
AK032815	Fusion, derived from t(12;16) malignant liposarcoma (human)	0.222	0.169	1.314

DNA microarray using fluorescent probes made with cDNAs from Th1 and Th2 cells. For this purpose, naïve CD4 T cells were isolated from the spleen of C57BL/6 mice and stimulated *in vitro* in either Th1 or Th2 polarizing conditions for 2 days. RNA isolated from naïve, Th1, or Th2 differentiated cells was used for making fluorescent probes. Fluorescent probes were hybridized to a DNA microarray, and the fluorescence intensity was measured in each spot of the microarray. The intensity of Th1 and Th2 probes normalized to that of naïve probes. Among 71 unique genes of nuclear receptors and coregulators, 20 and 26 genes were induced more than 2-fold compared with naïve cells in Th1 and Th2 cells, respectively. On the other hand, 38 and 40 genes were reduced more than 2-fold compared with naïve cells in Th1 and Th2 cells, respectively. By comparing the normalized intensity of genes between Th1 and Th2 cells, we identified genes that preferentially expressed in one subset (Table 2 and Table 3). From these genes, we selected genes, the expression of which was increased compared with naïve CD4 T cells and were selectively expressed in one subset more than 1.7-fold. One gene [RAR-related orphan receptor alpha (RORα, NM_013646] was found to be selectively expressed in Th1 cells, and three genes [retinoid X receptor alpha (RXRα, BC024493), RAR-related orphan receptor gamma (RORγ, NM_011281), and nuclear receptor coactivator 3 (NCoA3, NM_008679] were found to be selectively expressed in Th2 cells.

Confirmation of Differential Expression by RT-PCR and Western Blotting

We confirmed the selective expression of these nuclear receptors and coregulators by RT-PCR. Since many genes found by the microarray analysis are previously shown to be highly expressed in Th17 cells, in particular RORγt and RORα, we performed RT-PCR analysis including Th17 cells as well as Th1 and Th2 cells. Naïve CD4 T cells were stimulated *in vitro* in Th1, Th2, or Th17 polarizing conditions for 2 days, and RNA was isolated from the Th1, Th2, or Th17 cells and used for RT-PCR.

The RT-PCR showed similar expression patterns as the microarray data, validating the microarray data. As was previously shown by others [14, 39], RORα and RORγt were highly expressed in Th17 cells (Fig. 1A). The result also showed that retinoid X receptor α (RXRα) was highly expressed in Th2 cells (Fig. 1A). To validate the lineage-specific pattern of the cDNA sample used in this study, we also measured the expressions of IFN-γ, IL4, and IL17, which are specifically expressed in Th1, Th2, and Th17, respectively (Fig. 1A). These cytokines were expressed in a Th1-, Th2-, or Th17-specific manner as expected, confirming that RNA used in our study represent the proper differentiation status.

The differential expression of nuclear receptors and coregulators was also confirmed by Western blotting (Fig. 1B).

Table 3. Genes selectively expressed in Th2 cells identified by microarray.

GeneBank Acc. No.	Name	Th1/naïve	Th2/naïve	Th2/Th1
BC024493	Retinoid X receptor alpha	0.795	2.129	2.678
NM_011281	RAR-related orphan receptor gamma	5.000	9.500	1.900
NM_008679	Nuclear receptor coactivator 3	1.792	3.044	1.699
NM_009801	Carbonic anhydrase 2	0.177	0.266	1.503
BC076623	Nuclear receptor coactivator 7	1.735	2.514	1.449
AK040046	Thyroid hormone receptor associated protein 1	0.169	0.244	1.444
NM_017388	Eosinophil-associated, ribonuclease A family, member 2	0.124	0.173	1.395
NM_011750	Splicing factor 1	1.807	2.417	1.338
NM_009365	Transforming growth factor beta 1 induced transcript 1	0.330	0.434	1.315

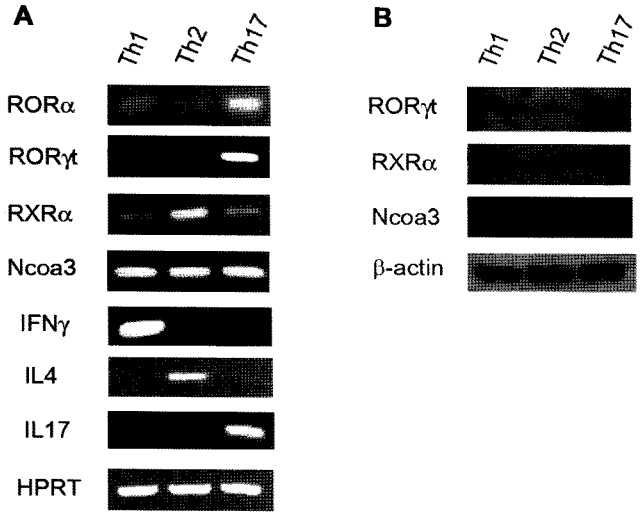

Fig. 1. Confirmation of Th1-, Th2-, and Th17-selective expression of genes by RT-PCR and Western blotting
Naïve CD4 T cells were *in vitro* differentiated to Th1, Th2, or Th17 for 2 days. **A.** RNA was extracted and reverse transcribed. cDNA were amplified by PCR with specific primers shown in Table 1. **B.** Cell extracts were prepared and subjected to Western blotting with specific antibodies.

Consistent with RT-PCR analysis, RORγt was highly expressed in Th17 cells, and RXRα was highly expressed in Th2 cells (Fig. 1B).

RORγ has two isoforms: the isoform RORγ1 is expressed in many tissues including liver and muscle, whereas RORγt is specifically expressed in only two cell populations, DP thymocytes and lymphoid tissue inducers (LTi) [5, 7]. The two isoforms differ only in their N-terminus. These two isoforms are generated by use of an alternative promoter within the second exon of the RORγ gene (resulting in a protein lacking the N-terminal 24 residues of RORγ) [12]. Since the RORγ gene in the microarray (Table 3) detects both isoforms of RORγ, that is RORγ1 and RORγt, we examined which isoform is expressed in T helper cells. We also examined whether different isoforms are expressed in different subsets of T helper cells. For this purpose, we performed RT-PCR with specific primers for RORγ1 and RORγt with RNAs isolated from Th1, Th2, and Th17 cells (Fig. 2). The result showed that RORγt but not RORγ1 is selectively expressed in Th17 cells (Fig. 2). The expression of RORγt is very weak in Th1 and Th2 cells and is negligible compared with Th17 cells (Fig. 2).

Selective Roles of Nuclear Receptors in the Regulation of Cytokine Genes Expression

Of the nuclear receptors and coregulators screened by microarray analysis and confirmed by RT-PCR, RXRα showed selective expression in Th2 cells. We further examined the function of nuclear receptors and regulators in the regulation of Th1 and Th2 cytokine gene expression. We transfected CMV-based expression constructs containing

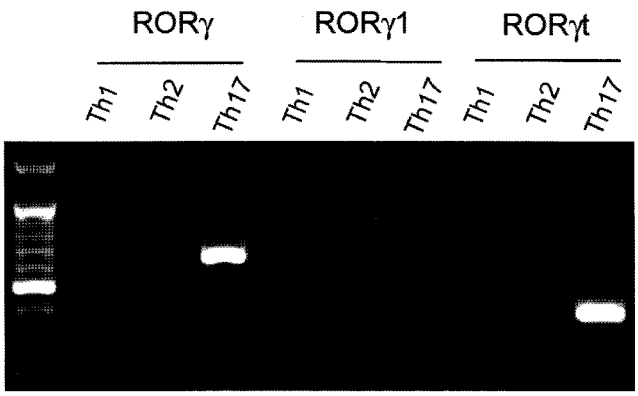

Fig. 2. RT-PCR with specific primers for RORγ(both), RORγ1, and RORγt.
Naïve CD4 T cells were *in vitro* differentiated to Th1, Th2, or Th17 for 2 days. RNA was extracted and reverse transcribed. cDNA were amplified by PCR with specific primers for RORγ(both γt and γ1), RORγ1, and RORγt.

various nuclear receptor and coregulator genes into Jurkat cells, a human T cell lymphoma cell line. After stimulation we isolated RNA from the transfected cells and measured IL4, IFN-γ and IL17 gene expression by RT-PCR. Overexpression of RXRα enhanced the expression of the IL4 gene but had no effect on IFN-γ or IL17 gene expression (Fig. 3). As expected, overexpression of RORα or RORγt induced IL17 expression in this system (Fig. 3). This result indicates

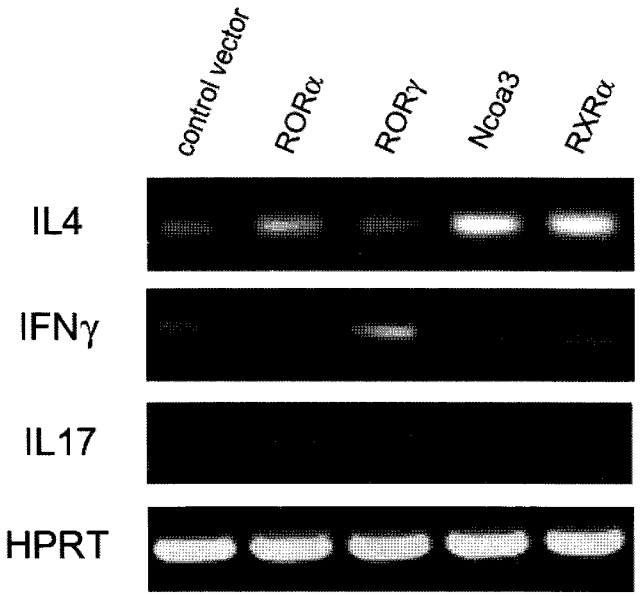

Fig. 3. Overexpression of RXRα-induced IL4 gene expression.
CMV-based expression constructs containing mRORα, mRORγ, mNcoA3, and hRXRα genes were transfected to Jurkat cells. After 16 h, cells were stimulated by PMA plus ionomycin and total RNA was isolated from the cells. Expression of the IL4, IFN-γ, and IL17 genes was measured by RT-PCR. Expression of the HPRT gene was used as a control. Experiments were repeated three times with similar results.

that RXRα has selective effect on the regulation of IL4 gene expression.

Discussion

By microarray analysis and RT-PCR, we screened nuclear receptors and coregulators that are selectively expressed in Th1 or Th2 cells. Our study showed selective expression and function of the nuclear receptor, playing a role in the regulation of Th1 and Th2 cytokine genes.

Our result shows that RXRα is highly expressed in Th2 cells compared with Th1 cells (Table 3 and Fig. 1). Overexpression of RXRα in Jurkat cells induced IL4 gene expression but had no effect on IFN-γ expression. This result is consistent with previous results that RXR is involved in Th2 cell differentiation [4, 9, 16, 25, 29–31]. RXR is activated by 9-*cis*-retinoic acid (9-*cis*-RA), a metabolite of vitamin A. Activation of RXR has been shown to inhibit IL12 production of macrophages and to promote Th2 differentiation *in vitro* [16, 25, 31]. *In vivo*, vitamin A-deficient mice are unable to mount an effective Th2-mediated immune response and launch an excessive Th1-response instead [2]. Disruption of RXRα in mice resulted in alteration of the balance of Th1/Th2 and caused exaggerated Th1 responses and reduced Th2 responses [4, 9, 29, 30]. RXR serves as a heterodimerization partner for a variety of other nuclear hormone receptors, including the VDR, the liver X receptor, and the PPAR. Our result that RXRα is highly expressed in Th2 cells and enhances IL4 gene expression sheds light on the mechanism of a differential effect of retinoic acid on T helper cell differentiation.

As was previously described, our result confirmed the selective expression of RORγt and RORα in Th17 cells (Fig. 2). The ROR family consists of three structurally related members, RORα (NR1F1), RORβ (NR1F2), and RORγ(NR1f3) [5]. They share a highly conserved DNA binding domain (DBD) located at the N-terminus and a less well conserved putative ligand binding domain (LBD) that is located at the C-terminus. The DBD of the RORs is approximately 66 amino acids long and most of the amino acids in this region are identical in these three members [11, 15]. In contrast, the LBD of RORs shares approximately 50% identity at the amino acid level. Although they do not share ligands, the highly conserved DBD of RORs suggests that these orphan receptors may bind to the same DNA elements.

In the immune system, RORα is expressed in both lymphoid and myeloid cells [6]. Macrophages have the highest level of RORα expression, followed by T cells and B cells. The initial studies on the *in vivo* function of RORα come from an animal model known as *staggerer* mice [28]. *Staggerer* mice contain a mutation with a 122-bp deletion in the sequence coding for the LBD of RORα that results in a frame-shift [10]. It was found that the size and cellularity of both the thymus and the spleen in *staggerer* mice were dramatically reduced when compared with heterozygous littermates [35]. Recently, the role of RORα in lymphocyte development and function was directly investigated in RORα KO mice as well as Rag-2 KO mice reconstituted with RORα KO bone marrow [6]. RORα KO T and B lymphocytes proliferate normally, but RORα KO CD8 T cells produce an increased amount of IFN-γ after TCR stimulation. Recently, Yang *et al.* [39] reported that RORα synergizes with RORγt to promote Th17 differentiation. They have shown that overexpression of RORα induced IL17 expression, and that RORα deficiency caused reduction of IL17 expression. RORα and RORγ double deficiencies completely impaired Th17 differentiation *in vivo* [39].

RORγt and RORγ1 inhibit TCR-mediated apoptosis in T cell hybridomas by inhibiting the expression of FasL. The importance of RORγ1 and RORγt in immune function has been demonstrated in mice lacking the expression of both isoforms (herein referred to as RORγ KO mice). Two major defects have been observed in RORγ KO mice. The first defect is an impaired thymocyte development and the second is a complete lack of secondary lymphoid organs with the exception of the spleen in the mutant mice [19, 33]. Recently, Ivanov *et al.* [14] have shown that RORγt is the critical transcription factor for differentiation of Th17 cells. Ectopic expression of RORγt induced IL17 production, and mice deficient in RORγt T cells have attenuated autoimmune disease and lack tissue-infiltrating Th17 cells [14].

In summary, this study searched for nuclear receptors and coregulators that are differentially expressed in Th1 and Th2 cells. This study found that RXRα is highly expressed in Th2 cells and that RXRα selectively regulates the expression of IL4. This study provides an insight to how the differentiation of the T helper cell might be regulated by nuclear receptors and stimulates further research in this field.

Acknowledgments

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korean government (MOST)(R01-2007-000-10211-0), and by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2006-331-C00214).

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