Review

The Roles of Orphan Nuclear Receptors in the Development and Function of the Immune System

Ivan Dzhagalov[1], Nu Zhang[1] and You-Wen He[1, 2]

Hormones and their receptors regulate cell growth, differentiation and apoptosis and also play important roles in immune function. Recent studies on the subfamily of the orphan nuclear receptors known as retinoid-acid related orphan receptors (ROR) have shed important insights on the roles of this group of nuclear proteins in the development and function of the immune system. RORα regulates inflammatory cytokine production in both innate and adaptive immune system while RORγ regulates the normal development of T lymphocyte repertoire and secondary lymphoid organs. *Cellular & Molecular Immunology*. 2004;1(6):401-407.

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Introduction

The nuclear hormone receptor superfamily is composed of both ligand-regulated transcription factors as well as orphan receptors (1, 2). These receptors perform diverse functions in development, reproduction, and homeostasis by regulating cell growth, differentiation, and apoptosis. Aberrant activities of certain receptors have been causally linked to neoplasia. Despite an intensive search for their ligands, most members of the orphan nuclear receptor family still do not have an identified ligand (3). In fact, some of the orphan receptors appear to function independent of a natural ligand. Recent works on two subfamilies of the orphan nuclear receptors, the ROR subfamily and the Nur77 subfamily, have shed important new insights on their roles in the development and function of the immune system. This review will focus on the recent advances made on the ROR subfamily.

The orphan nuclear receptor ROR subfamily consists of three structurally related members, RORα (NR1F1) (4, 5), RORβ (NR1F2) (6), and RORγ (NR1F3) (7-9). 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 (10, 11). In contrast, the LBD of RORs shares approximately 50% identity at the amino acid level. Though they do not share ligands, the highly conserved DBD of RORs suggests that these orphan receptors may bind to the same DNA elements.

RORα

RORα was initially discovered through its homology to retinoid acid receptor (RAR). It is widely expressed in a variety of tissues, with particularly high levels in cerebellum, skin, testis and peripheral blood leukocytes (12-14). In the immune system, RORα is expressed in both lymphoid and myeloid cells (15). Macrophages have the highest level of RORα expression, followed by T cells and B cells. Four isoforms of RORα have been isolated in human, RORα1-4 (5, 6), while only RORα1 and RORα4 have been detected in mouse (12, 13). These isoforms, generated through alternative splicing and different promotor usage, have unique DNA binding abilities. The differences between them lie solely in the N-terminus of the molecule. All RORα isoforms bind either to the consensus site AGGTCA preceded by 6 A/T rich base pairs or to a direct repeat of the consensus sequence separated by 2 nucleotides (DR2) (16, 17). The differences in the N-termini of the RORα isoforms and their cooperation with the DBD and the C-terminal extension of the molecule determine the exact binding specificity of each isoform. Consistent with its broad expression pattern, RORα binding sites can be found in the regulatory regions of many genes. However, direct transcriptional regulation by RORα has only been shown for a few genes including N-myc, prosaposin, ApoA-1, Purkinje cell protein 2 (PCP2) and Rev-erb (18-23).

[1]Department of Immunology, Duke University Medical Center, Durham, NC 27710, USA.

[2]Corresponding to: Dr. You-Wen He, Box 3010, DUMC, Durham, NC 27710, USA. Tel: +01-919-613-7870, Fax: +01-919-684-8982, E-mail: he000004@mc.duke.edu.

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Abbreviations: ROR, retinoid-acid related orphan receptor; LBD, ligand binding domain; DBD, DNA binding domain; RAR, retinoid acid receptor.

The molecular mechanism by which RORα operates is not entirely clear. Studies have implicated that RORα interacts with both nuclear co-repressors to repress gene expression as well as co-activators to activate it (24, 25). To date, most identified target genes controlled by RORα are positively regulated by this orphan receptor, reinforcing the idea that RORα is a constitutive activator of gene transcription.

The regulation of RORα activity may depend on other signaling molecules. It has been shown that RORα activity is Ca^{2+} responsive. Furthermore, a Ca^{2+}/calmodulin-independent form of CAMK IV enhances the transcriptional activity of RORα (26). However, CAMK IV does not directly phosphorylate RORα, suggesting that it acts on co-factor involved in RORα-mediated activity.

Whether RORα has a ligand is a matter of debate. Melatonin was reported as a possible natural ligand for RORα (27). However, further evidence is needed to support this. A more likely ligand for RORα is cholesterol. Recently, Kallen et al. reported the crystal structure of RORα (28). Surprisingly, this group found a small molecule in the ligand-binding pocket of RORα which they identified as cholesterol. Cholesterol derivatives such as cholesterol sulfate appear to have higher affinity than cholesterol itself and are able to displace it from the ligand-binding pocket of RORα (29). In fact, mutations of RORα that prevent binding of cholesterol decrease RORα-mediated transcriptional activity in an osteosarcoma cell line.

Additional support for cholesterol as a ligand for RORα was provided by the development of RORα deficient mice. These mice develop severe atherosclerosis when fed high fat diets (30). The major factor for atherosclerosis is hypercholesterolemia, and RORα has been shown to regulate the expression of Apolipoprotein A (ApoA) (19). The idea that a ubiquitously present molecule such as cholesterol or its derivatives serves as a natural ligand for RORα is attractive since RORα is known to constitutively activate target genes. Although it is possible that cholesterol only functions to stabilize RORα, the prevailing view is that RORα will soon be deorphanized.

The initial studies on the *in vivo* function of RORα came from an animal model known as *staggerer* mice (31). *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 (12, 32). These mice display a complex phenotype with abnormalities in the development and function of multiple organs and usually die between 3 and 4 weeks of age. The most obvious defect in *staggerer* mice is their balance deficit due to massive neurodegeneration in the cerebellum, which is caused by a developmental defect in Purkinje cells. Other abnormalities include osteoporosis, muscular atrophy and susceptibility to atherosclerosis when fed atherogenic diets (16, 33). The bones of *staggerer* mice are unusually long and fine and their mineral content is decreased (osteopenia) (34). In fact, RORα appears to regulate two important genes in bone mineralization-syaloglycoprotein and osteocalcin. The phenotypes exhibited by *staggerer* mice suggest that RORα plays critical roles in the development and maintenance of bone tissue, muscles and osteoporosis.

All the defects found in *staggerer* mice were recapitulated in RORα knockout ($ROR\alpha^{-/-}$) mice, indicating that the *staggerer* phenotype is indeed a result of the lack of RORα function (35, 36). Interestingly, some of the phenotypes exhibited in *staggerer* mice are a little bit more severe than in $ROR\alpha^{-/-}$ mice. For example, $ROR\alpha^{-/-}$ mice are less asthenic and they reach better equilibrium scores and perform significantly better in the rotarod test than *staggerer* mice (36). The differences in the phenotypes may be caused by the nature of the mutations in the two types of mice. In $ROR\alpha^{-/-}$ mice the DBD is disrupted while in *staggerer* mice the LBD is truncated. As a result, in $ROR\alpha^{-/-}$ animals no DNA binding capacity is present, while in *staggerer* mice the DBD of RORα is preserved and may function as a dominant negative inhibitor.

An early study on *staggerer* mice suggested that RORα plays a critical role in lymphocyte function (37). It was found that the size and cellularity of both the thymus and the spleen in *staggerer* mice were dramatically reduced when compared to heterozygous littermates. A prolonged immune response to sheep red blood cells (SRBCs) was also demonstrated. However, these studies did not define the nature of the defects in lymphocyte development and function. Recently, the role of RORα in lymphocyte development and function was directly investigated in $ROR\alpha^{-/-}$ mice as well as $Rag\text{-}2^{-/-}$ mice reconstituted with $ROR\alpha^{-/-}$ bone marrow (15). $CD4^+CD8^+$ double positive (DP) thymocytes are almost completely absent from the thymus of $ROR\alpha^{-/-}$ mice with the majority of the cells being $CD4^+$ or $CD8^+$ single positive (SP). B lymphocyte development in the BM is disrupted in a very similar manner: the immature B cells ($CD43^-B220^+IgM^+IgD^-$) are almost completely missing while the mature B cells (IgM^+IgD^+) are present. However, the severely impaired T and B lymphocyte development in $ROR\alpha^{-/-}$ mice is not lymphocyte intrinsic since T and B lymphocytes develops normally in $Rag\text{-}2^{-/-}$ mice reconstituted with $ROR\alpha^{-/-}$ BM. The defective lymphocyte development in $ROR\alpha^{-/-}$ mice might be caused by stress due to body imbalance of these mice. Alternatively, the neurological defect in $ROR\alpha^{-/-}$ mice may result in abnormal activities of hormones that can affect lymphocyte development.

Due to early lethality of the $ROR\alpha^{-/-}$ mice, the function of $ROR\alpha^{-/-}$ lymphocytes was assessed in $Rag\text{-}2^{-/-}/ROR\alpha^{-/-}$ BM chimeric mice (15). Interestingly, serum IgG levels are elevated in $Rag\text{-}2^{-/-}/ROR\alpha^{-/-}$ chimeric mice after immunization with a T-dependent antigen when compared with control chimeras. The increased antibody production is likely an indirect effect from cytokines. Although $ROR\alpha^{-/-}$ T and B lymphocytes proliferate normally, $ROR\alpha^{-/-}CD8^+$ T cells produce an increased amount of IFN-γ after TCR stimulation (15). Furthermore, $ROR\alpha^{-/-}$ mast cells and macrophages produce a dramatically increased amount of TNF-α and IL-6 upon stimulation of TLR4 by LPS. The higher level of these cytokines in $ROR\alpha^{-/-}$ chimeric animals may promote antibody class-switching by B lymphocytes and explain the increased IgG levels after immunization.

These results demonstrate that RORα differentially regulates cytokine production in T lymphocytes and mast

cells and macrophages. It acts as a negative regulator of IFN-γ but does not affect production of IL-6 and TNF-α in $CD8^+$ T cells. It does, however, act as a negative regulator of IL-6 and TNF-α in mast cells and macrophages (15, 38, 39). The molecular mechanisms by which RORα regulate cytokine production is currently under investigation. Multiple transcription factors including NF-κB, NF-AT, T-bet, and Eomesodermin are involved in the regulation of IFN-γ production in T lymphocytes (40-43). A previous report showed that over-expression of RORα in smooth muscle cells inhibits TNF-α-induced expression of IL-6, IL-8, and cyclooxygenase-2, and upregulates the expression of IκBα (44). RORα may negatively regulate cytokine production by activating the transcription of IκBα, which in turn inhibits the activation of NF-κB. Alternatively, RORα may interact with other transcription factors that are involved in the production of IFN-γ, IL-6 and TNF-α.

What is the physiological role of RORα in a normal immune system? The expression of RORα in macrophages is tightly controlled. It is highly expressed in resting macrophages and down-regulated upon Toll-like receptor (TLR) ligand stimulation (15). This expression pattern of RORα suggests that one role of RORα is to prevent resting macrophages from producing inflammatory cytokines. However, RORα expression is not down-regulated in activated $CD4^+$ and $CD8^+$ T lymphocytes, suggesting a continued requirement for the presence of this orphan receptor to regulate their function. In further support of this, $ROR\alpha^{-/-}$ T lymphocytes exhibit increased response to a model pathogen, *Listeria monocytogenes* (15). Thus, the role of RORα in T lymphocytes may be to down-size the activated antigen-specific $CD8^+$ T cell response.

RORβ

In contrast to the other two members of the ROR family, RORβ has a restricted expression pattern. It is detected only in the central nervous system (CNS), retina, epididymis and vas deferens (45-47). In the CNS, RORβ is expressed in areas involved in processing sensory information including the spinal cord, thalamus and sensory cerebellar cortices. In addition, this orphan receptor is expressed in the major regions of the mammalian timing system - the suprachiasmatic nuclei, the retina and the pineal gland. The distribution of RORβ suggests that it is involved in the processes of sensory information integration and circadian rhythm. Indeed, young mice deficient for RORβ exhibit muscular weakness and walking abnormalities (48). In addition, adult $ROR\beta^{-/-}$ mice display a "duck-like" gate and a hind paw clasping reflex when suspended by the tail. Histological examination of $ROR\beta^{-/-}$ retina revealed that it is severely malformed and degenerated. As a consequence, $ROR\beta^{-/-}$ mice are blind.

Recently, all-trans retinoic acid (ATRA) has been proposed as RORβ ligand after a co-crystal complex was reported (49). Competition binding assays showed a high affinity interaction between ATRA and RORβ (Kd = 280 nM). Moreover, RORβ transactivation was strongly inhibited by retinoids, suggesting that the role of ATRA is to inhibit the constitutively activated RORβ. RORβ may cross-talk with RARs since RARs use ATRA as their ligand (50). Interestingly, while ATRA activates transcription after binding to RAR, it appears to suppress RORβ-mediated transcription after binding RORβ (48). The *in vivo* relevance of ATRA in regulating RORβ function remains to be established. As RORβ expression is largely restricted to the CNS, its role in the development and function of the immune system remains to be analyzed.

RORγ

The third member of the ROR family, RORγ, has attracted intense attention from immunologists recently. RORγ has two isoforms: the isoform RORγ is expressed in many tissues including liver and muscle (7, 8), while RORγt (51), is specifically expressed in only two cell populations, DP thymocytes and lymphoid tissue inducers (LTi) (51-53). The two isoforms differ only in their N-terminus. RORγ has a longer N-terminus that is coded by the first two exons of the locus. RORγt N-terminus is encoded by an exon that lies after the first two RORγ exons and it uses a separate promoter (54). Both isoforms share the same exons for the DBD and LBD. The DBD and LBD of RORγ are highly conserved among different species. In contrast to RORα and RORβ, there have been no ligands described so far for RORγ. RORγ was first isolated in degenerate PCR or low-stringency hybridization using the DBD of the nuclear receptor family as a searching template (7, 8). RORγ contains 516 amino acids with an estimated molecular mass of 58 kD. RORγt was cloned in a retrovirus-based functional screening to identify genes involved in CD3/TCR-mediated cell death. RORγt contains 495 amino acids (51). RORγt and RORγ inhibit TCR-mediated apoptosis in T cell hybridomas by inhibiting the expression of FasL.

The importance of RORγ and RORγt in immune function has been demonstrated in mice lacking the expression of both isoforms (herein referred as $ROR\gamma^{-/-}$ mice). Two major defects have been observed in $ROR\gamma^{-/-}$ 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 (55, 56). RORγt appears to regulate thymocyte maturation at several distinct stages. Thymocyte development in $ROR\gamma^{-/-}$ mice is severely blocked at the transition from the immature single positive (ISP) stage ($CD4^-CD8^+TCR^-$) to DP stage (57). Mice deficient for two other transcription factors, the E-box protein HEB and the target of Wnt signaling TCF-1, exhibit a similar blockade at this transition (58, 59). It will be interesting to determine the relation between RORγt and these transcription factors. Another major defect in $ROR\gamma^{-/-}$ mice is the massive apoptosis of DP cells. Together with the blockade at ISP stage, the increased DP apoptosis in $ROR\gamma^{-/-}$ mice may account for their dramatically decreased thymic cellularity. The increased cell death of DP cells in $ROR\gamma^{-/-}$ mice is likely due to a decreased expression of the anti-apoptotic protein Bcl-xL in these cells (55, 56) . When $ROR\gamma^{-/-}$ mice were crossed with a Bcl-xL transgenic line, the massive

cell death of DP cells was restored to normal levels. Clearly, Bcl-xL is a target gene controlled by RORγt. However, RORγt might regulate Bcl-xL expression indirectly since there are no identifiable RORγ/γt binding sites in the regulatory regions of Bcl-x gene. Although RORγt inhibits FasL expression in T cell hybridomas, the massive apoptosis of DP cells in RORγ$^{-/-}$ mice is not due to a de-repressed expression of FasL since crossing RORγ$^{-/-}$ mice into *gld* background (FasL mutant) does not rescue the cell death (51, 56). Aside from the increased cell death, a large fraction of DP thymocytes from RORγ$^{-/-}$ mice enter the cell cycle (56). This is in sharp contrast to the quensicent status of normal DP thymocytes. Since Bcl-xL trangene expression also corrects the cell cycle defect of RORγ$^{-/-}$ mice, this defect appears to result from decreased Bcl-xL expression. In support of this view, Bcl-xL has been shown to inhibit cell cycle progression (60, 61).

Another important function of RORγ/γt is to regulate TCRα repertoire by virtue of its positive regulatory role on Bcl-x expression. RORγt has been shown to bind to the TEA promoter in the Jα locus of TCRα genes and thus has been suggested to regulate TCRα recombination (54). When the TCRα repertoire was examined in the RORγ$^{-/-}$ mice, it was surprising that the Jα usage was skewed to the 5' end of the locus (57). This defect contrasts the impaired 5' Jα usage found in TEA-deficient mice. Furthermore, the impaired 3' Jα usage in RORγ$^{-/-}$ mice was corrected by the expression of Bcl-xL as a transgene. Strikingly, transgenic expression of Bcl-xL not only corrected defective 3' Jα usage in thymocytes of RORγ$^{-/-}$ mice, but also skewed Jα usage to the very 3' end of the locus in both RORγ$^{-/-}$ and wild-type mice. Moreover, Bcl-xL transgene expression induced a 3' Jα bias to the peripheral TCRα repertoire as well. These results demonstrate that programmed cell death of DP thymocytes is an important parameter that limits the progression of rearrangements along the Jα locus and regulates the repertoire of positively selected T cells.

RORγt also plays an essential role for the development of secondary lymphoid organs as RORγ$^{-/-}$ mice completely lack lymph nodes (LN) and Peyer's patches (55, 56). Lymphoid organogenesis proceeds in several distinct steps. In the initial stage, a population of hematopoietic origin that is CD4^{+}CD3^{-}CD45^{+}IL-7Rα^{+} colonizes LN and Peyer's patch anlagen and serves as lymphoid tissue inducer (LTi) (62, 63). RORγt but not RORγ is expressed in LTi cells that are absent in the RORγ$^{-/-}$ mice (52). RORγt expressing LTi cells can be detected in tight clusters in the lymph node anlagen as early as E12.5. Colonizing LTi cells interact with stromal cells expressing ICAM-1 and VCAM-1, and activate them through LTαβ_2-LTR pathway to produce chemokines that attract lymphocytes (64). Although it is clear that RORγt is essential for the generation of LTi cells, the function of RORγt in the generation of this population remains to be determined. Lack of lymphoid organs in RORγ$^{-/-}$ mice cannot be restored by a Bcl-xL knock-in in the RORγt locus, arguing against a role of RORγt in protecting LTi cells from apoptosis (52). Furthermore, although injection of LT-α agonistic antibody rescues the development of lymph nodes in LTα$^{-/-}$ mice, it has no effect in RORγ$^{-/-}$ mice, suggesting the defect in lymphoid organ development in RORγ$^{-/-}$ mice is not due to a lack of LT signaling (52, 65). Interestingly, mice lacking the inhibitor of E-box proteins Id2 exhibit a strikingly similar defect in their lymphoid organ development (66). The molecular relation between these molecules in lymphoid organogenesis deserves further investigation.

RORγt regulates lymphocyte homeostasis by controlling the normal development of splenic microenvironment. Recent studies by Zhang et al. demonstrate that the spleen of RORγ$^{-/-}$ mice is enlarged 2-3-fold compared to littermate controls due to an accumulation of conventional resting B lymphocytes (67). The increased number of B cells in the spleen of mutant mice is not caused by abnormal B cell development or proliferation. The peripheral T cell compartment in RORγ$^{-/-}$ mice is also apparently normal. Interestingly, when wild-type T and B cells derived from transferred bone marrow were examined in the RORγ$^{-/-}$ hosts, these cells also accumulated in the spleen. These results indicate that the splenic microenvironment of RORγ$^{-/-}$ mice is defective. How does RORγt regulate splenic microenvironment if it is not detected in the spleen? The answer is that RORγt is expressed in the spleen during early embryogenesis (53). One might speculate that RORγt regulates stromal differentiation in fetal spleen. This observation is interesting since it suggest that lymphocyte migration out of secondary lymphoid organs is actively regulated.

Recently Littman and co-workers tracked the origin of intraepithelial lymphocytes (IELs) using a genetic mapping approach based on the fact that RORγt is only expressed in DP thymocytes but not in other T cell compartments (68). The origin of IEL has been studied extensively and conflicting results suggest that IEL may develop either intrathymically or extrathymically (69, 70). To track developing T lymphocytes that have gone through the thymus, a mouse line with inserted GFP under a floxed stop codon was crossed with a mouse line expressing the Cre recombinase under the promoter of RORγt. Any cells that have gone through DP thymocyte stage will be permanently marked with GFP expression. Using this elegant approach, it has been shown that all the IELs are marked with GFP and thus derived intrathymically.

Although recent works have clearly established the critical roles of the orphan receptor RORγ/γt in T cell development and lymphoid organogenesis, many questions remain to be answered. For example, what are the target genes for RORγ/γt? Bcl-xL appears to be an indirect target in DP thymocytes. Other transcription factors such as Stat6, glucocorticoid receptor and NF-κB have been demonstrated to play roles in the regulation of Bcl-xL expression (71-73). Does RORγt interact with these transcription factors?

The initial observation that RORγt protects T cell hybridoma from TCR-mediated apoptosis may provide some mechanistic insights on its function (51). RORγt inhibits the upregulation of both FasL and IL-2 in T cell hybridoma cells. Since both FasL and IL-2 promotors are positively regulated by nuclear factor of activated T cells (NF-AT), RORγt may inhibit NF-AT function specifically in DP thymocytes.

Indeed, expression of RORγt inhibits an NF-AT-binding luciferase reporter in Jurkat cells (74). Moreover, RORγt is able to bind to an NF-AT binding site and competes with NF-AT. So, the increased apoptosis of DP

thymocytes in the absence of RORγt might be due to uncounteracted NF-AT activity (75, 76). Although the expression of FasL is not de-repressed in DP thymocytes of RORγ$^{-/-}$ mice, making it an unlikely player in the premature death of the DP cells, NF-AT is still an attractive target for RORγ. The restricted expression of RORγt in DP thymocytes may function to displace NF-AT from its target genes since NF-AT is indispensible for T cell proliferation and DP thymocytes are the only population in the thymus that does not divide. Moreover, ectopic expression of RORγt inhibits the proliferation of mature T cells and DN thymocytes (77). Second, NF-AT, together with NF-κB and AP-1, is the major factor regulating the transcription of many cytokine genes. While both DN and SP thymocytes secret cytokines when stimulated, DP thymocytes do not (78). In addition, forced expression of RORγt in mature T cells prevents them from producing IL-2 (77). Thus, a critical role of RORγt in DP cells is to impose quiescent phenotype on DP thymocytes before the cells are positively selected.

In summary, recent studies from our group and others have demonstrated important functions of orphan nuclear receptors in the development and function of the immune system. Given the increasing number of orphan receptors that have been identified, the roles of orphan receptors in immune function will continue to shed novel insights on the fundamental processes of immunity.

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