European Journal of Immunology Highlights

COMMENTARY:

Twisting the Th1/Th2 immune response via the retinoid X receptor: Lessons from a genetic approach

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Received 11/10/05
Accepted 2/11/05

[DOI 10.1002/eji.200535588]

The immune system is influenced by environmental factors such as hormones and nutrients. Previous studies have suggested that vitamins A and D influence the process of naive T helper (Th) cell differentiation into Th1 or Th2 cells. Vitamins A and D signal through the retinoid X receptor (RXR), which partners with either the retinoic acid receptor or the vitamin D receptor. Most previous studies into the role of RXR in Th differentiation have been performed *in vitro* and it was necessary for these to be verified in a physiological environment. However, *in vivo* study has been hindered since RXRα deficient mice are embryonic lethal. Du *et al.* in this issue of the *European Journal of Immunology*, overcome this obstacle using "*pinkie*" mice that harbor a hypomorphic mutation in the *Rxra* gene. The authors report that the mutant mice have an exaggerated Th1 immune response which is attributed to the aberrant antigen-presenting cell and CD4 T cell function. This study confirms previous studies indicating that RXR signaling plays an important role in Th cell differentiation and also provides a valuable tool with which to study the mechanisms and the *in vivo* functions of this signaling pathway.

Key words: Allergies · $CD4^+$ cells · Retinoid receptors · *pinkie* mouse · Th1/2 cell differentiation

See accompanying article: http://dx.doi.org/10.1002/eji.200535366

One of the major health problems in most advanced Western countries in the past few decades has been the increased incidence of allergic diseases. This is the case for type I (atopic) diseases such as bronchial asthma, allergic rhinoconjunctivitis ("hay fever") and atopic eczema/dermatitis, while there has been no similar increase for other allergic diseases such as type II (cytotoxic), type III (immune complex) or type IV (delayed type) hypersensitivity reactions such as allergic contact dermatitis. "Atopy" is the genetic predisposition determining the susceptibility to develop atopic diseases. The more we know about the genes involved in atopic diseases, in combination with the characterization of key mutations in these genes, the more it becomes clear that genetic factors are not the only reason for the increase observed, and that environmental factors are also implicated. Such factors include changes in eating habits, the increased indoor exposure

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Abbreviations: **RA:** retinoic acid · **RAR:** RA receptor · **RXR:** retinoid X receptor · **vitD3:** vitamin D3 · **VDR:** vitamin D receptor

to allergens because of poor living conditions and the increased exposure to pollen due to atmospheric pollution. Since allergies concern predominantly the immune system, it is noteworthy that the continuous exposure to infectious diseases of either viral, bacterial, fungal or parasitic origin that has been strongly reduced in modern Western society, but which was a hallmark of the past, has been proposed to explain the dramatic increases in asthma – the so called hygiene hypothesis (reviewed in [1]).

The immune system protects our body from invading foreign pathogens and perhaps, to an extent, from cancer. The immune system is not only activated by pathogens and cancers but is also influenced by the environment of the body *e.g.* by hormones and nutrients. Accumulating evidence suggests that nutrient status is an important factor contributing to immune competence [2]. Nutrients demonstrated to be required for the immune system to function include essential amino acids, fatty acids and vitamins. Among these, vitamins A and D have been implicated in the differentiation of Th1/Th2 cells [3], cells which constitute the adaptive arm of the immune response and mediate a variety of immune responses.

One of the major processes that influences the adaptive immune response is Th cell differentiation. The balance of Th1 and Th2 cell populations is of great importance for the generation of allergies and specific immune responses and this balance is influenced by environmental factors. Naive $CD4^+$ cells have undergone positive selection prior to their exit from the thymus to the periphery but have not yet encountered cognate antigen. Naive $CD4^+$ T cells can follow at least two differentiation pathways: they can differentiate into either Th1 cells producing the signature cytokine IFN-γ or Th2 cells producing the signature cytokines IL-4, IL-5 and IL-13. Th1 cells are responsible for protection against intracellular pathogens while Th2 cells are protective against extracellular parasites.

The activation of the *Il-4* locus is dependent on the expression of the transcription factor GATA3 [4], whereas the initial transcriptional activation of the *Ifn-γ* gene depends on the expression of the transcription factor T-bet [5]. Upon activation of $CD4^+$ naïve T cells through T cell antigen receptor signaling, subsequent lineage commitment and expansion of Th1 and Th2 cells requires signaling through the IL-12 and IL-4 receptors respectively. Upon IL-12 signaling, STAT4 phosphorylation and activation leads to heritable expression of the *Ifn-γ* gene in Th1 cells and the subsequent repression of the *Il-4* gene. On the other hand, IL-4 signaling leads to phosphorylation and activation of the transcription factor STAT6 which leads to heritable transcriptional activation of the *Il-4* gene and the subsequent suppression of the *Ifn-γ* gene (Fig. 1a). The development of $CD4^+$ T cells into either Th1 or Th2 cells determines the outcome of an immune response and, as discussed, is primarily directed by cytokines; however, in parallel with the major role of cytokines in Th cell development and differentiation, other factors have also been described that affect the Th1/Th2 balance, such as the spectrum of TCR and costimulatory signals or the antigen dose. Also a number of molecules, such as vitamins glucocorticoids and eicosanoids, have been described which influence Th cell development [6].

A number of different experimental approaches have shown the diverse effects of vitamins, especially vitamin A (retinol), in the regulation of different biological pathways such as vertebrate development, cellular differentiation and homeostasis. There are different levels of regulation to form such a diverse output of retinoid signals. The effects of vitamin A are primarily mediated via its retinoic acid (RA) derivatives, including the all-*trans*- (atRA) and 9-*cis* (9cRA) forms. Mouse genetic studies support the notion that a major mechanism underlying the diverse responses upon retinoid signaling depends on the utilization of two

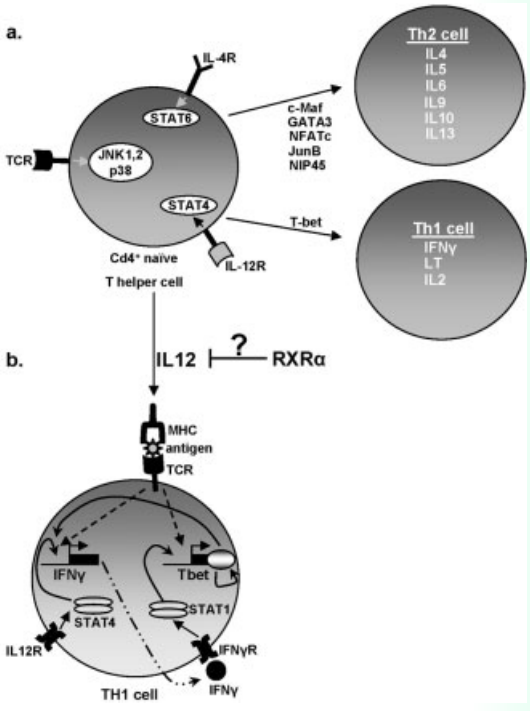

Figure 1. $CD4^+$ Th cell differentiation. (a) The major signals (TCR+IL-12R and TCR+IL-4 signalling) and transcription factors (STAT4 and STAT6) implicated in the differentiation processes of naive $CD4^+$ T cells to Th1 and Th2 cells respectively are depicted. In addition, the cytokines produced from each cell lineage are indicated. (b) A detailed analysis of the signals and transcription factor networks that lead to differentiation of Th1 cells. It is possible that RXRα impairs Th1 differentiation by blocking IL-12 signaling. Whether other pathways are involved requires further experimental work and the present uncertainty regarding this matter is depicted by "?".

Table 1. Retinoic acid and retinoid X receptors

Gene	Chromosome		Isoforms	Ligand
	mouse	human		
RARα	11	17q21.1	α1, α2	all trans and 9-cis RA
RARβ	14	3p24	β1, β2, β3, β4	all trans and 9-cis RA
RARγ	15	12q13	γ1, γ2	all trans and 9-cis RA
RXRα	2	9q34.3	α1, α2	9-cis RA
RARβ	17	6p21.3	β1, β2	9-cis RA
RARγ	1	1q22-q23	γ1, γ2	9-cis RA

different families of receptors, the RAR and the RXR families, and the fact that they can function as heterodimers and directly bind to multiple DNA response elements [7]. Like RAR, RXR can also form homodimers which can positively regulate transcription. The RAR and RXR families possess the conserved domain structure typical of nuclear receptors and their amino acid sequence can be divided into six regions (A–F) according to their homology with other nuclear receptors [8].

The vitamin A derivatives atRA and 9cRA regulate gene transcription by binding to either α, β, or γ RAR or α, β, or γ RXR (Table 1). Both atRA and 9cRA bind to the RAR, whereas 9cRA can also act via RXR. RAR and RXR belong to a family of nuclear receptors that also includes the vitamin D receptor (VDR), the thyroid hormone receptor (TR), and the peroxisome proliferation/activation receptor (PPAR), the latter binding specific fatty acids. The RXR/RAR heterodimers bind to their target regulatory sites and recruit a number of corepressor or coactivator proteins [8, 9]. These coactivators induce chromatin remodeling through intrinsic histone-modifying activities and direct recruitment of key transcription initiation components to regulated promoters. Since RXR forms heterodimers with RAR, VDR, PPAR and TR, RXR is critically involved in both the vitamin A and D signaling pathways, and many other pathways.

The outcome of the immune response can be regulated by modifying the balance of Th1 and Th2 cells. Th cell differentiation is directly dependent not only on antigen stimulation but also on the cytokine levels that promote a certain Th cell lineage. Vitamin A deficiency increases constitutive IL-12 production by macrophages, and during secondary *in vitro* stimulation of lymphocytes with Ag, vitamin A deficiency increases IFN-γ production, but decreases IL-4 and IL-5 production. On the other hand, supplemental treatment with vitamin A or RA decreases IFN-γ but increases IL-5, IL-10, and IL-4 production [10]. Thus, a vitamin A deficiency biases the immune response in a Th1 direction, whereas high-level dietary vitamins may bias the response in a Th2 direction. Vitamin D3 (vitD3) has been shown to regulate the differentiation, growth, and function of a broad range of cells, including cells of the immune system, as well as mineral and skeletal homeostasis. VitD3 has been shown to regulate the immune system by affecting the activity of transcription factors, such as the NF-AT, NF-κB, and SMAD transcription factor families [11, 12]. Activated macrophages possess the enzyme 1-α-hydroxylase that allows for the production of vitD3, which suggests a role for this endogenously produced steroid hormone in the regulation of immune responses. RA appears to be the metabolite of vitamin A that is most potent in restoring impaired Ab responses. Although it is known that exogenous RA can down-regulate IFN-γ transcription, little else is known about how RA modulates the Th1/Th2 balance.

To unravel the mechanisms of the mode of action of vitamin A and its derivatives, a targeted *Rxrα* gene deletion in mice was constructed; however, the resultant RXRα-deficient mice were embryonic lethal between days 13.5 and 16.5 due to heart failure [13]. Therefore, to examine the influence of RA on Th cell differentiation, studies have mostly been performed in vitamin A deficient animals or by utilizing *in vitro* systems involving administration of retinoids into cell cultures. To unravel the *in vivo* role of vitamin A, different systems such as conditional knockout technology in mice were required.

In their study in this issue of the *European Journal of Immunology*, Beutler and colleagues [14] have shed light on some of the issues discussed above. The authors induced random germline mutagenesis using N-ethyl-N-nitrosourea and identified a viable hypomorphic allele of *Rxrα* ($Rxr\alpha^{Pke}$). The phenotype of the mice containing this mutant allele, called *pinkie*, is complete hair loss (alopecia) at four months of age, and the development of cysts under the ventral skin. The phenotype is more severe in female compared to male mice and by one year of age a fraction of the mutant mice develop dorsal kyphosis, the appearance of "dry" eyes and corneal

opacity. The *pinkie* phenotype was mapped to chromosome 2 and was identified as a single nucleotide change in the third α-helix of the ligand binding domain of the *Rxrα* gene. The mutant receptor loses 90% of its activity in both RA and vitamin D signaling pathways, as shown by transient transfection assays, and the phenotype cannot be rescued by exogenous addition of vitamins.

Exploring the immune response of the mutant mice, the authors found that one year old mice have a significant impairment of antigen-specific IgG1 production implying an impairment in Th2 responses. When $CD4^+$ naive T cells were stimulated under Th1 polarizing or mixed Th1/Th2 conditions mutant cells produced considerably higher amounts of IFN-γ compared to IL-4. No differences between mutant and wild type IL-4 producing cells were observed when Th2 polarizing conditions were used for the differentiation of naive cells. Two important conclusions can be drawn from these experiments. First, RXRα signaling normally suppresses the differentiation of naive $CD4^+$ T cells into Th1 cells and, second, RXRα indirectly permits Th2 differentiation of naive cells but is not directly required for this process. Previous reports have suggested that vitD3 inhibits Th1 differentiation [15] but the effect of vitD3 on Th2 differentiation was found to be somewhat variable with VitD3 either reducing or increasing this process [16]. This issue has been clarified by the study of Beutler and colleagues [14] and although their study could not distinguish between the pathways for vitamins A and D, stimulating the converging pathways of these vitamins clearly resulted in the inhibition of Th1 differentiation and an increased Th2 response.

Consistent with the dependency of Th cell differentiation on the abundance of specific cytokines, which is controlled at the transcription level by transcription factors, $Rxr\alpha^{Pke}$ naive cells when differentiated under mixed Th1/Th2 conditions expressed more Tbet mRNA (necessary for the expression of IFN-γ and the differentiation of Th1 cells) and less GATA3 mRNA (necessary for the expression of IL-4 and differentiation of Th2 cells). Furthermore the authors found that mutant dendritic cells produced higher levels of IL-12 with or without stimulation by lipopolysaccharide. The important conclusion that can be drawn from this result is that the RXRα signaling pathway normally suppresses IL-12 production in dendritic cells independently of TLR4 signaling and this suppressive action is independent of IL-10, since wild type and mutant dendritic cells showed no differences in IL-10 production under the above experimental conditions.

In addition to T cell activation, other data suggests that vitD3 controls regulatory T cell functions [17]. One of the ways that vitD3 represses T cell function is through APC. VDR agonists arrest the differentiation and maturation of APC by maintaining them in an immature state manifested by low expression of costimulatory molecules, decreased IL-12 production and increased IL-10 production [18]. VitD3, however, also directly affects T cell function. It has been reported that in an APC-free *in vitro* system repeated stimulation of $CD4^+$ T cells in the presence of vitD3 and corticosteroids (dexamethasone) resulted in a population of cells predominantly producing IL-10 and with the ability to suppress autoimmune demyelination *in vivo* in an antigen-specific manner [16]. Beutler and colleagues [14] report that blocking RXR signaling results in the decreased function of regulatory T cells, supporting a role for the RXR pathway (probably *via* vitD3) in the generation of regulatory T cells. It will be of interest to ascertain the mechanism whereby RXR influences the suppressive function of regulatory T cells in a Foxp3 and IL-10 independent manner.

Since *Rxrα* deficient mice are not viable, most studies conducted to delineate the mechanism of the mode of action of vitamin A and its derivatives on the differentiation of helper T cells have been performed *in vitro*. *In vitro* studies are informative but lack the complex environment and influence of multiple factors of an *in vivo* model. Beutler and colleagues [14] by utilizing the mutagenic approach of introducing a point mutation and changing the binding affinity of RXRα were able to directly investigate the regulatory functions of the vitamin A signaling pathway *in vivo*. From these studies, the clear conclusion that the RXRα signaling pathway is necessary for Th2 responses *in vivo* can be drawn. Consistent with previous studies, *Rxrα* deficiency leads to a defect in naive $CD4^+$ T cells and dendritic cells which compromises Th2 differentiation and contributes to Th1 polarization.

During the differentiation of Th1 cells from naive $CD4^+$ T cells, IFN-γ production is induced by IL-12 through phosphorylation and dimerization of STAT4 [19]. The Th1 specific transcription factor Tbet is then induced by IFN-γ through phosphorylation and dimerization of STAT1 which also upregulates IL-12R expression in naïve $CD4^+$ T cells [20]. A question that requires further experimentation is whether IL-12 inhibition by RXRα is the sole inhibitory pathway leading to negative regulation of Th1 differentiation or whether other pathways are also involved in this process (Fig. 1b).

Acknowledgements: C.S. is supported with a Cancer Research Institute fellowship; R.A.F. is an Investigator of the Howard Hughes Medical Institute.

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