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Regulation of CD8⁺ T cell functions by RAR γ

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Abstract

Retinoic acid plays a key role in the development and function of the immune system; however, the contribution of each of the three retinoic acid receptors (RARs) to the T cell immune response is not yet well understood. Of these receptors, both RAR α and RAR γ are expressed in T lymphocytes. While possible functional redundancy thus complicates understanding of the role of each receptor in T cells, emerging data suggest that RAR α and RAR γ function differently in thymocyte development and that RAR γ is required for both primary and secondary CD8⁺ T cell immune responses.

Keywords

Retinoic acid receptor γ ; CD8⁺ T lymphocytes

1. Introduction

The role of vitamin A in immunity has long been appreciated, with reports as early as the 1920s that vitamin A deficiency causes atrophy of lymphoid organs [1]. The importance of vitamin A in preventing infection has been recognized in numerous studies, and vitamin A supplementation is considered one of the major public health measures worldwide [2]. That vitamin A derivatives (referred to as retinoids) can modulate cell-mediated immunity was reported in the 1970s; however, the complex mechanisms by which retinoids regulate T cell development and function are only beginning to be elucidated [3]. This review will focus on the effects of retinoids, and in particular, retinoic acid receptor γ , on CD8⁺ T cells.

2. RAR and RXR families

Vitamin A (retinol) is intracellularly metabolized to form compounds known as retinoids. Derivatives of vitamin A, including all-trans-retinoic acid and 9-cis-retinoic acid, are recognized by members of two families of nuclear receptors: the retinoic acid receptors (RARs) and retinoic acid X receptors (RXRs) [4]. The three members of the RAR family, RAR α , RAR β , and RAR γ , are encoded by distinct genes, and bind both all-trans-retinoic acid and 9-cis-retinoic acid, while members of the RXR family bind only 9-cis-retinoic acid [5–9]. Each of the three RAR subtypes are present as multiple isoforms differing in the 5' untranslated region and N terminal A region generated from differential promoter usage and alternative mRNA splicing [10–15].

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Heterodimers formed between RARs and RXRs regulate transcription in a ligand-dependent manner. RAR/RXR heterodimers constitutively bind retinoic acid response elements (RAREs), and in the absence of ligand, recruit corepressor proteins to inhibit transcription of target genes [16–26]. Upon RAR ligand binding, corepressors are displaced, allowing heterodimers to instead recruit coactivators, resulting in transcriptional activation [24–26].

3. RAR expression in lymphocytes

During embryonic development, RAR α is nearly ubiquitously expressed, while the expression patterns of RAR β and RAR γ are much more limited [27,28]. In adult mice and humans, RAR γ expression is largely restricted to the skin, while RAR β is expressed in the cerebral cortex, prostate, and kidneys [29–33].

The expression of the various RAR isoforms has been examined in human and mouse lymphocytes and is summarized in Tables 1 and 2. RAR α 1 and RAR γ 1 are constitutively expressed in human T and B cells, while RAR β 2 expression is only detected following treatment with all-trans-retinoic acid [34]. RAR γ 2 is expressed only at low levels in human lymphocytes, and its expression is not induced by all-trans-retinoic acid, while RAR β 1 and RAR β 3 are not expressed in T or B cells under any conditions [34]. Similarly, RAR α and RAR γ , but not RAR β , are expressed by T cell hybridomas as well as murine thymocytes and thymic stromal cells [35–37]. Expression of RAR α begins in thymocytes by day 17 of embryonic development, while RAR γ is not expressed, or is expressed only at very low levels prior to birth [38].

Postnatal RAR γ expression is differentially regulated throughout T cell development. CD4⁺ CD8⁺ double positive (DP) thymocytes express low levels of RAR γ [39]. While expression increases in both CD4⁺ and CD8⁺ single positive (SP) thymocytes, CD8⁺ SP thymocytes express 2–3 fold more RAR γ than CD4⁺ SP thymocytes [39]. Expression continues to increase in mature CD4⁺ T cells; however, their expression level remains less than that of mature CD8⁺ T cells [39].

4. RAR γ and T Cell Development

Vitamin A deficiency has long been known to cause thymic atrophy [1]. Further evidence for a role for retinoids in the thymus is provided by the observation that endogenous RAR ligands are generated from vitamin A in the thymus through a retinaldehyde dehydrogenase (RALDH)-dependent metabolic pathway [40]. Based on the results of both *in vitro* and *in vivo* studies, several potential functions for retinoic acid in thymocyte development have been proposed.

First, retinoic acid inhibits TCR-mediated thymocyte apoptosis *in vitro* [41]. In agreement with this finding, retinoic acid has been shown to inhibit negative selection of thymocytes. All-trans-retinoic acid or 9-cis-retinoic acid supplementation results in the development of increased numbers of SP thymocytes in fetal thymic organ culture [38]. This increase in mature thymocytes appears to be due to inhibition of negative selection, as all-trans-retinoic acid supplementation blocked superantigen-mediated deletion of CD4⁺ V β 3⁺ thymocytes and partially prevented the SEB-mediated deletion of V β 6⁺ and V β 8⁺ thymocytes [38]. Similarly, retinoic acid inhibits negative selection of H-Y TCR transgenic thymocytes cultured with male thymic stromal cells, with 9-cis-retinoic acid showing a 10-fold more potent effect than all-trans-retinoic acid [42].

These effects appear to be mediated by an RAR α /RXR-dependent pathway, and antagonized by an RAR γ -dependent pathway. The greater potency of 9-cis-retinoic acid suggests that RXRs may be the relevant receptor in inhibiting thymocyte death; however, although addition of an RXR-specific agonist decreased the concentration of all-trans-retinoic acid required for

inhibition of apoptosis, very high doses of this agonist were required to replicate the effects of 9-cis-retinoic acid [43]. A role for RAR α was demonstrated by the ability of RAR α -specific agonists to efficiently inhibit activation-induced thymocyte apoptosis both *in vitro* and *in vivo* [43]. Furthermore, addition of RAR α -specific antagonists blocked the protective effects of retinoic acid [43]. RAR γ -specific agonists, on the other hand, enhanced activation-induced thymocyte apoptosis, and RAR γ -specific antagonists reduced the concentration of all-trans-retinoic acid necessary for inhibition of apoptosis [43]. Together, these results suggest that RAR α is required for retinoic acid-mediated inhibition of apoptosis, and that RXR ligand binding counteracts antagonism from RAR γ .

The ability of RAR γ to cause thymocyte apoptosis has been documented both *in vitro* and *in vivo*. In contrast to observations in TCR-stimulated thymocytes, addition of either all-trans-retinoic acid or 9-cis-retinoic acid to unstimulated cultures of either whole thymocytes or sorted DP thymocytes results in increased apoptosis [36]. This increase in apoptosis appears to be mediated by RAR γ , as RAR γ -specific agonists, but not RAR α -specific agonists were able to replicate the effects of retinoic acid [36]. The ability of an RAR γ -mediated pathway to cause thymocyte apoptosis was confirmed *in vivo*, as treatment of mice with an RAR γ -specific agonist resulted in rapid thymic involution largely due to loss of DP thymocytes [36].

The mechanism by which RAR γ enhances apoptosis has not been examined in thymocytes. However, studies in T cell hybridomas suggest that RAR γ ligation results in nur77-mediated upregulation of cell surface FasL, while RAR α ligation inhibits FasL expression by blocking the ability of nur77 to bind DNA, and also inhibits activation-induced upregulation of the proapoptotic Bcl2 family member Bim [37,44,45].

The role of RAR γ in thymocyte development has been further examined using genetic models allowing either overexpression or deletion of RAR γ . Notably, no changes in thymic cellularity or apoptotic rates were reported in either model, possibly due to differences in concentration of exogenous ligand used in previous studies from physiologic levels present in the thymus [39,46].

Mice expressing a human RAR γ transgene in T cells display alterations in thymocyte development, with an increase in CD8 T cells in both the thymus and the periphery [46]. These results, along with the increased expression of RAR γ observed in CD8⁺ SP thymocytes, suggest that RAR γ may play a role in the CD4/CD8 lineage decision. However, T cell development is unaffected in the absence of RAR γ . Mice lacking RAR γ in hematopoietic cells have normal thymic cellularity, and the frequency of DN1-DN4, DP, and CD4⁺ and CD8⁺ SP thymocytes are similar to those in wild type mice [39]. Furthermore, normal numbers of mature CD4⁺ and CD8⁺ T cells were observed in the periphery [39]. While these data indicate that RAR γ is not required for normal T cell development, it is possible that the presence of RAR α may compensate for loss of RAR γ in some functions. To better understand the function of each receptor in T cell development, analysis of RAR α -deficient and RAR α /RAR γ double-deficient mice, along with *in vivo* administration of selective inhibitors of each RAR will be necessary.

5. RAR γ and the CD8 T Cell Response

Early studies demonstrated that although high doses of retinoic acid cause a dramatic reduction in thymic and splenic cellularity, lower doses enhance cell-mediated immune responses [3]. *In vivo* treatment with low doses of retinoic acid resulted in a tenfold increase in cytotoxicity of splenocytes, but did not enhance mitogen-induced splenocyte proliferation [3]. While the functions of retinoic acid in cell-mediated immunity have not been completely elucidated, RAR γ has been implicated in promoting the CD8⁺ T cell response.

Although the absence of RAR γ has no effect on T cell development, CD4⁺ T cell polarization, or T-dependent humoral immune response, mice lacking RAR γ in hematopoietic cells (referred to hereafter simply as RAR γ -deficient) mount a defective CD8⁺ T cell response to *L. monocytogenes* infection [39]. Seven days following infection with OVA-expressing *L. monocytogenes* (rLmOVA), significantly fewer Ifn γ ⁺ antigen-specific CD8⁺ T cells were present in RAR γ -deficient mice than in wild type mice [39]. Additionally, splenocytes from rLmOVA-immunized RAR γ -deficient mice displayed decreased cytotoxicity when compared to splenocytes from immunized wild type mice [39]. However, it is not clear whether the decreased cytotoxicity observed in total splenocytes is due simply to the decreased number of antigen-specific CD8⁺ T cells in these mice, or loss of cytotoxic function on a per cell basis in antigen-specific CD8⁺ T cells in the absence of RAR γ .

The CD8⁺ memory response was also found to be impaired in the absence of RAR γ . The numbers of both Ifn γ ⁺ and antigen-specific CD8⁺ T cells present after rechallenge were significantly lower in mice lacking RAR γ in hematopoietic cells than those in wild type mice [39]. These effects were specific to CD8⁺ T cells, as the numbers of Ifn γ ⁺ antigen-specific CD4⁺ T cells were similar to those in wild type mice both at the peak of the primary response and after secondary challenge [39].

The cause of impaired effector and memory CD8⁺ T cell induction in the absence of RAR γ remains unclear. It is possible that RAR γ may play a role in inducing production of Ifn γ in CD8⁺ T cells (Fig. 1A). However, the role of retinoic acid generally, and RAR γ specifically in Ifn γ expression is unknown. While supplementation with all-trans-retinoic acid enhances Ifn γ production in LPS-challenged rats, *L. monocytogenes*-infected rats receiving all-trans-retinoic acid supplementation produce less Ifn γ than unsupplemented rats [47,48]. Furthermore, no RAR γ binding sites were identified in the promoter region of the Ifn γ gene, suggesting that RAR γ could only indirectly regulate transcription of Ifn γ [39]. Additionally, no defect in Ifn γ production was observed in RAR γ -deficient Th1-polarized CD4⁺ T cells, indicating that any requirement for RAR γ must be CD8⁺ T cell-specific [39].

As RAR γ was deleted in all hematopoietic cells, it is not clear whether the observed defects are intrinsic to CD8⁺ T cells, or due to changes in other cells of the immune system. For example, macrophages in RAR γ -deficient mice produce lower levels of inflammatory cytokines after TLR stimulation (Fig. 1C) [39]. However, serum levels of inflammatory cytokines during early infection were normal in these mice, and *S. typhimurium*, GBS, and *L. monocytogenes* infections were cleared effectively, indicating that the decreased CD8⁺ T cell response is not likely caused by a defect in the innate immune response [39].

That CD8⁺ T cells develop normally in mice lacking RAR γ in hematopoietic cells but show functional defects in the periphery could be indicative of defects in hematopoietic-derived antigen presenting cells that are not present in non-hematopoietic antigen presenting cells, such as thymic epithelial cells. Reports that expression of MHC class I may be regulated by retinoic acid raise the possibility that CD8⁺ T cells may fail to respond to infection due to a defect in antigen presentation in the periphery of RAR γ -deficient mice (Fig. 1B).

A novel RARE has been identified in the downstream responsive element of the mouse H2Kb gene and found to be highly conserved in MHC class I genes [49]. The outer repeat sequences are present and unmutated in all 16 classical MHC class I genes in mice, and the intact RARE is also conserved in other mammalian species [49].

9-cis-retinoic acid was found to activate transcription of a reporter containing this RARE [49]. Furthermore, RAR/RXR heterodimers were shown to both bind this RARE, and to activate reporter transcription in the presence of ligand [49]. While RAR β 2/RXR γ , RAR α 1/

RXR γ , RAR α 1/RXR α , and RAR β 2/RXR α heterodimers were most effective, all heterodimers were able to activate transcription [49].

However, whether MHC class I expression is regulated by retinoic acid *in vivo* is unknown. Additionally, although *in vitro* studies show that the three RAR subtypes are differentially expressed in human cord blood monocytes, monocyte-derived dendritic cells, and macrophages, the expression patterns of RARs in antigen presenting cells *in vivo* has not been examined [50]. As the ability to mount an effective CD8⁺ T cell response to *L. monocytogenes* is critically dependent on cross-presentation of antigens by CD11c⁺ dendritic cells, studies of the roles of RARs in dendritic cell function may prove particularly informative [51].

Thus, while it is clear that the CD8⁺ T cell response is impaired in mice lacking RAR γ throughout the hematopoietic system, further experiments are necessary to determine which cell populations require RAR γ and which gene products are regulated by RAR γ during both primary and secondary immune responses.

6. RAR γ and T lymphocyte gut homing

In addition to appropriate T cell activation, an effective CD8⁺ T cell response requires homing of T cells to sites of infection. The roles of retinoic acid in imprinting tissue tropism have been best studied in the context of CD8⁺ T cell gut homing; however, the mechanisms by which retinoic acid may mediate T cell homing to gut-associated lymphoid tissue (GALT) are not entirely clear.

The ability of antigen-experienced T cells to home to the gut is mediated in part by α 4 β 7 integrin and CCR9 [52,53]. The ligands for these receptors, MAdCAM-1 and the chemokine CCL25, are expressed by high endothelial venules in mucosa, and by epithelial cells of the small intestine, respectively [54–57].

MadCAM-1, which preferentially interacts with α 4 β 7 over α 4 β 1 integrin, has been shown to play a role in lymphocyte gut homing [54,55]. That MadCAM-1 mediates lymphocyte extravasation into mucosal tissue was first demonstrated by experiments showing that treatment of frozen mesenteric lymph nodes and Peyer's patches, but not peripheral lymph nodes, with MadCAM-1-blocking antibodies prevented lymphocytes isolated from mesenteric lymph nodes from binding HEVs [56]. This function was confirmed *in vivo*, as treatment of mice with anti-MadCAM-1 antibody completely inhibited lymphocyte homing to Peyer's patches, partially inhibited homing to mesenteric lymph nodes, but did not affect homing to peripheral lymph nodes [56]. That MadCAM-1-dependent gut homing is mediated by its interaction with α 4 β 7 integrin was demonstrated by studies showing that pretreatment of lymphocytes with anti- α 4 β 7 Fab fragments prior to adoptive transfer inhibits migration to Peyer's patches [58]. In contrast, blocking of the α 4 β 1 integrin-specific ligands VCAM-1 and fibronectin had no effect on lymphocyte homing to Peyer's patches, further suggesting that gut homing depends specifically on α 4 β 7 integrin [58].

CCR9 is expressed by most naïve CD8⁺ T cells, but only half of recently activated CD8⁺ T cells, and a small population of previously activated CD8⁺ T cells [53]. Immunization results in sustained upregulation of CCR9 in antigen-specific CD8⁺ T cells in mesenteric but not peripheral lymph nodes, and 40–50% of intestinal epithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) were found to be CCR9⁺ [53]. That CCR9 expression is required for migration of antigen-specific CD8⁺ T cells to the gut has been demonstrated by *in vivo* neutralization of CCL25. Treatment with a neutralizing anti-CCL25 antibody prior to immunization results in a significant reduction in the number of antigen-specific CD8⁺ T cells

in the small intestinal epithelium, but does not affect the frequency of antigen-specific CD8⁺ T cells in spleen, mesenteric, or peripheral lymph nodes [53].

The expression and function of CCR9 in CD4⁺ T cells is less clear. Some studies have shown a small population of CCR9⁺ CD4⁺ T cells, while others do not detect CCR9 expression in CD4⁺ T cells [52,53]. As in CD8⁺ T cells, CCR9 is selectively upregulated in antigen-specific CD4⁺ T cells activated in mesenteric lymph nodes; however, CCR9-deficient antigen-specific CD4⁺ T cells are able to migrate to the lamina propria, though less efficiently than wild type cells [52].

The gut tropism of CD8⁺ T cells activated in GALT appears to be a function of the antigen presenting cells present in these tissues, as dendritic cells from Peyer's patches and mesenteric lymph nodes, but not peripheral lymph nodes or spleen induce upregulation of CCR9 and $\alpha 4\beta 7$ integrin [59,60]. *In vitro* experiments have shown that dendritic cells from Peyer's patches and mesenteric lymph nodes, but not spleen, convert all-trans-retinol to all-trans-retinoic acid, and the presence of either all-trans-retinoic acid or Am80, which preferentially activates RAR α , results in enhanced expression of $\alpha 4\beta 7$ integrin and CCR9 in T cells [61,62]. Further, treatment with the RAR antagonist LE135 suppresses the ability of Peyer's patches and mesenteric lymph node dendritic cells to upregulate $\alpha 4\beta 7$ integrin and CCR9 in T cells, while the RXR pan-antagonist PA452 has no effect, suggesting that RARs, but not RXRs are required for T cell gut tropism [61]. LE135 has been used as an RAR β -specific antagonist; however, in some studies, it has been shown to also act on RAR α [63,64]. As T cells do not express RAR β , these data suggest that RAR α activation plays a role in induction of $\alpha 4\beta 7$ integrin and CCR9 expression.

The function of RAR γ in T cell gut tropism was examined in mice lacking RAR γ in hematopoietic cells. Upon anti-CD3 stimulation in the presence or absence of all-trans-retinoic acid, RAR γ -deficient CD4⁺ and CD8⁺ T cells upregulated $\alpha 4\beta 7$ to levels similar to those in control T cells.[39] In addition, similar frequencies of TCR $\alpha\beta$ ⁺, TCR $\alpha\delta$ ⁺, CD4⁺ CD8⁻, CD4⁻ CD8 α ⁺, and CD4⁺CD8⁺ IELs were observed in wild type and RAR γ -deficient mice, suggesting that all-trans-retinoic acid-dependent T cell gut tropism does not require RAR γ [39]. However, as mature T cells express both RAR α and RAR γ , it is possible that RAR α is able to compensate for the loss of RAR γ . To investigate this possibility, it will be necessary to compare the ability of T cells to home to the gut in RAR α /RAR γ double deficient mice to that in RAR α and RAR γ single deficient mice.

8. Summary

Understanding of the role of RAR γ in CD8⁺ T cell function is complicated by the expression of both RAR α and RAR γ in developing and mature T cells. A role for RAR γ in the CD8⁺ T cell response has been demonstrated *in vivo*; however, it is unclear whether RAR γ is required by T cells or by antigen presenting cells. *In vitro* experiments have shown that RAR γ can mediate thymocyte apoptosis; however, no pro-apoptotic function has been observed *in vivo*. Further, whether RAR γ influences the CD4⁺ vs. CD8⁺ lineage decision in the thymus remains unclear. Finally, although no role for RAR γ has been demonstrated in CD8⁺ T cell gut homing, it is possible that RAR α may compensate for the loss of RAR γ . In order to fully elucidate the functions of RAR γ , future studies will need to address possible redundancy between members of the RAR family as well as determine which cell populations truly require RAR γ expression.

Abbreviations

RAR

retinoic acid receptor

RXR	retinoic acid X receptor
RARE	retinoic acid response element
DP	CD4 ⁺ CD8 ⁺ double positive
SP	single positive
RALDH	retinaldehyde dehydrogenase
DN	double negative
rLmOVA	OVA-expressing <i>L. monocytogenes</i>
IEL	intestinal epithelial lymphocyte
LPL	lamina propria lymphocyte

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Figure 1. Possible functions of RAR γ in the CD8⁺ T cell response

A. Ligation of RAR γ may result in increased production of Ifn γ in CD8⁺ T cells. This may occur directly through RAR γ -regulated transcription of Ifn γ (top); however, the lack of any identifiable RAR binding sites in the Ifn γ promoter region suggests that RAR γ may instead upregulate an intermediate product that in turn mediates an increase in Ifn γ production (bottom).

B. RA-bound RAR γ /RXR heterodimers may promote transcription of MHC class I through previously identified RAREs in the MHC class I promoter region. Increased MHC class I expression may facilitate antigen cross-presentation for CD8⁺ T cell activation.

C. RAR γ is required for upregulation of inflammatory cytokines upon TLR stimulation of macrophages. RAR γ -mediated cytokine production is antagonized by all-trans-retinoic acid; however, it is unclear which receptor or receptors are responsible for this antagonism. As serum levels of inflammatory cytokines are normal in the absence of RAR γ , it is unclear whether RAR γ -mediated production of these cytokines is necessary for an efficient CD8⁺ T cell response.

Table 1

Expression of RAR α , RAR β , and RAR γ in thymocytes, peripheral T cells, and thymic stromal cells.

	RAR α	RAR β	RAR γ
Fetal thymocytes	+	—	—
Postnatal thymocytes	+	—	+
Mature CD4 T cells	+	—	+
Mature CD8 T cells	+	—	+
Thymic stroma	+		+

Table 2
Relative expression levels of RAR γ throughout T cell development.

	DP Thymocytes	CD4 ⁺ SP Thymocytes	CD8 ⁺ SP Thymocytes	Mature CD4 ⁺ T Cells	Mature CD8 ⁺ T Cells
RAR γ	+	++	++++	+++	++++