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Retinoic Acid in the Immune System

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Abstract

On occasion, emerging scientific fields intersect and great discoveries result. In the last decade, the discovery of regulatory T cells (T^{reg}) in immunity has revolutionized our understanding of how the immune system is controlled. Intersecting the rapidly emerging field of T^{reg} function, has been the discovery that retinoic acid (RA) controls both the homing and differentiation of T^{reg}. Instantly, the wealth and breadth of knowledge of the molecular basis for RA action, its receptors, and how it controls cellular differentiation can and will be exploited to understand its profound effects on T^{reg}. Historically, vitamin A deprivation and repletion and RA agonists have been shown to profoundly affect immunity. Now these findings can be interpreted in light of the revelations that RA controls leukocyte homing and T^{reg} function.

Keywords

vitamin A; retinoic acid; immune system; tolerance

Introduction

Dietary vitamin A deficiency and its impact on human biology has been a subject of intense scientific investigation for several decades. The absence of vitamin A from the body leads to the subsequent depletion of its downstream derivatives retinol and retinoic acid (RA), the alcohol and acid form of vitamin A, respectively. The absence of RA over the course of normal human development leads to defects in the immune system, embryonic development, vision, brain function, and other systems. All-*trans*-RA and 9-*cis* RA are the metabolically active derivatives of vitamin A and function as potent regulators of gene expression. They exert profound influence on leukocyte proliferation and differentiation.¹ The vital roles that vitamin A and RA play in the homeostatic control of the immune system have been known for decades, given the observation that vitamin A-deficient individuals are incapable of controlling bacterial, viral, and protozoan diseases.² Recent reports of the essential role of RA in maintaining gut lymphocyte homing and T regulatory cell differentiation have brought this small metabolite to the forefront as a critical regulator of immunity. Given the current availability of reagents and genetic approaches to elegantly manipulate RA activity and signaling *in vivo*, the stage is set for the immunologic community to explore the remarkable role of vitamin A in the immune system at an unprecedented resolution.

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Conflicts of Interest

The authors declare no conflicts of interest.

In this review, we will focus on the biology of all-*trans* RA and its impact on the immune system. In particular, we will highlight new developments identifying all-*trans* RA as a T regulatory cell differentiation factor and the implications this observation has for both oral and peripheral tolerance as well as for autoimmunity. As the basic biology of all-*trans* RA and its receptors has been reviewed elsewhere in detail, we include a brief introduction to these concepts because a basic understanding of the enzymatic machinery generating all-*trans* RA and the proteins involved in its transport and signal transduction is essential for a comprehensive discussion of the influence this metabolite has on the immune system.

Synthesis, Transport, and Signal Transduction of Retinoic Acid

Enzymatic Control of the Production of Retinoic Acid from Vitamin A

A series of enzymes are responsible for the metabolism of vitamin A to a complex variety of metabolites, with the final product in the pathway being RA. Vitamin A exists in the peripheral blood and liver primarily as retinol and retinyl esters, respectively, and these precursors must be enzymatically catalyzed by retinoid dehydrogenases into first retinal and then RA. The catalysis of retinol occurs in a reversible reaction by the retinol dehydrogenase enzymes that are members of the alcohol dehydrogenase (ADH) family. The ADH family has specificities for ethanol, retinoids, and other alcohols and aldehydes of physiological importance, and is responsible for the catabolism of retinal from retinol.¹ This reaction occurs within the cell cytosol and is driven by the ADH family members ADH1, ADH2, ADH3, and ADH4.³⁻⁵ The short-chain dehydrogenase/reductase (SDR) family, which shows a wide affinity for alcohols and aldehydes, has also been implicated in the catalysis of retinal from retinol.⁶ The final biosynthesis of retinal to retinoic acid occurs in an irreversible reaction driven by the aldehyde dehydrogenase (ALDH) family, of which there are three members in the mouse capable of driving the final step of RA synthesis.¹ These enzymes are known in mouse as Raldh1 (also Aldh1 or Aldh1a1), Raldh2 (otherwise known as Aldh1a2), and Raldh3 (also known as Aldh1a3).^{1,7,8} Highly homologous enzymes of both the ADH and ALDH family that serve similar enzymatic functions are present in humans and other mammals, indicative of the redundancy of this pathway due to the essential nature of RA metabolism to the organism.

The physiological importance of the ADH and RALDH enzymes and their respective contributions to RA production has been implicated through genetic deletion experiments in mice. While it was initially thought that redundancy between ADH1 and ADH4 in the metabolism of retinol to retinal may occur, intercrossing of ADH1-null and ADH4-null mice demonstrated distinct roles for these enzymes in retinal generation *in vivo*. The enzyme ADH4 plays an essential and exclusive role in retinal conversion during instances of dietary vitamin A deficiency (VAD) during development in a tissue-specific manner yet plays a minor role in the systemic oxidative retinol turnover pathway.⁹ The role of systemic generation of retinal is partially provided by ADH1, whose expression predominates in liver, the major retinol storage organ, and other tissues.^{3,9} ADH1 was not found to play a role in supporting retinal generation during developmental VAD, rather, this enzyme is thought to minimize vitamin A toxicity through elimination of excess retinol. To provide the body with systemic retinol, the enzyme ADH3 has been ascribed this role, with this enzyme providing both systemic retinal generation and protection during developmental VAD.^{4,5} While exhibiting inferior enzymatic activity compared to ADH1 and ADH4, the ubiquitous expression profile across all tissue types and the coupling of ADH3 enzyme expression with Raldh2, an enzyme essential for the second step of RA synthesis, has situated ADH3 as the major enzyme responsible for systemic RA production. The deletion of the gene encoding ADH3 produced mice with impaired RA generation *in vivo*, growth deficiency, and highly penetrant postnatal lethality during VAD.^{4,5}

Physiological contributions of the enzymes responsible for the second step of RA synthesis, the oxidation of retinal to RA, have been teased out by generating mice deficient in these enzymes. The *Raldh1*-null mouse showed no disturbance in overall viability yet implicated *Raldh1* in playing a major role in the production of all-*trans* RA in the liver.¹⁰ Analysis of the *Raldh2*-null mouse indicated that this enzyme plays an essential role in development due to the early lethality observed.^{11,12} A similar situation of developmental lethality was observed in the generation of the *RALDH3*-null mouse.¹³ The *in vivo* correlation of both ADH and RALDH mRNA and protein expression with all-*trans* RA production has afforded invaluable insights into the temporal and tissue-specific production of all-*trans* RA during embryonic development and in the biology of the immune system. In relation to the gut immune system being potently regulated by all-*trans* RA, the first insight that all-*trans* RA production can occur in the gut originated from a report describing high levels of ADH1 protein expression in the small and large intestines.⁹ The distribution of different RALDH isoenzymes in hematopoietic cells, like dendritic cells (DC), is likely key to determining the way RA controls leukocyte homing and function (discussed later).

Retinoid Binding Proteins: Vessels for Transport and Storage

Due to their lipophilic character, retinoids are not found in a free form in the body. Rather, they are associated with proteins specialized in the stabilization and delivery of retinoids to specific target tissues. Once retinoids are proximal to a cell, they associate with the membrane and are transported and stored in a complex with intracellular proteins.

There are three main families of retinoid binding proteins: retinol binding protein (RBP), cellular retinol (or retinal) binding protein (CRBP), and cellular retinoic acid binding protein (CRABP).⁶ RBP binds retinol when it is released from storage into the circulation, and as the main storage organ of retinol is the liver, this is the organ with the highest expression of RBP.¹⁴ The expression and release of RBP complexed with retinol depends on dietary vitamin A sufficiency; thus, upon vitamin A deficiency, RBP expression is decreased and its release to the circulation is inhibited.¹⁴ Genetic deficiency of RBP in mice demonstrated a requirement for RBP for the maintenance of normal levels of retinol in the blood as these mice were deficient in serum levels of retinol and exhibited impaired retinal function.¹⁵ RBP-null mice also demonstrated a larger storage of retinol in the liver in comparison with normal mice, implying a role for RBP in retinol transport from the liver to target tissues.¹⁵

Within the cell, all-*trans* retinol and all-*trans* RA bind two small intracellular proteins each exhibiting two isoforms that are members of the family of intracellular lipid-binding proteins. CRBP exists in two isoforms (CRBP-I and -II), with both able to bind all-*trans* retinol and all-*trans* retinal.^{6,14} The expression patterns of CRBP-I and CRBP-II are different, with CRBP-I expressed in liver, kidney, brain, and epithelia, and CRBP-II expressed at high concentrations in the small intestine.^{14,16} The principal function of CRBPs is to regulate the synthesis of RA from retinol, and it has been proposed that through retinol and/or retinal binding, CRBP-I controls the free concentration of the molecules by limiting the amount of substrate available and thus controlling downstream RA production.¹⁷ CRBP-II additionally regulates the access of retinol and retinal to their metabolic enzymes, but due to the limited expression of CRBP-II, it is thought that this protein may control the first steps of dietary retinol uptake from food.¹⁸

CRABPs are also found as two isoforms with all-*trans* RA their high-affinity ligand. A differential cell-specific pattern of expression is observed between the two isoforms of CRABPs. CRABP-I is expressed ubiquitously, while CRABP-II expression is limited to the ovaries, uterus, and skin, and correlates in expression to cell types that secrete large amounts of all-*trans* RA.¹⁴ These two all-*trans* RA binding proteins exhibit distinct functions, with CRAB-I modulating all-*trans* RA bioavailability by enhancing its metabolism and tempering

its intracytosol presence, and CRABP-II delivering all-*trans* RA directly to the nuclear RA Receptors (RARs), thus allowing its capture by the RARs.¹⁹ Once delivered to the RARs by holo-CRABP-II, all-*trans* RA complexed to RARs can mediate profound biological effects on cellular differentiation, proliferation, and apoptosis.

Retinoic Acid Nuclear Receptors

All-*trans* RA and 9-*cis*-retinoic acid bind to the RAR nuclear receptors, of which there are two types: RAR and RXR. These holo-complexes act as transcription factors binding to specific DNA sequences in the genome driving the transcription of target genes. The RAR family contains three members: RAR α (isoforms 1–2), RAR β (isoforms 1–4), and RAR γ (isoforms 1–2). Ligands of the RAR family can be either all-*trans* RA or its isomer 9-*cis* RA. The RXR family also contains three members (RXR α , - β , and - γ) and interacts at the physiological level only with 9-*cis* RA.²⁰ Upon encounter with all-*trans* RA, RAR heterodimerizes with RXR and binds retinoic acid response elements (RARE) in the genome, and 9-*cis* RA induces homodimerization of RXR.²¹

To dissect the role of each of these nuclear receptors at the whole-organism level, mice genetically deficient in a number of these nuclear receptors have been produced. Mice lacking RAR α , RAR β , RAR γ , or RXR α are phenotypically normal.²¹ Null mutants in pan-RAR or RAR α show defects similar to those observed in mice with a postnatal VAD such as pre- and postnatal development malformations, male sterility, and photoreceptor degeneration.^{22,23} To bypass the developmental defects manifest in these pan-RAR and pan-RAR α null strains, dominant negative RAR α (dnRAR α) has been created.^{24,25} The dnRAR α still binds to DNA and heterodimerizes with RXR, but either cannot bind co-activators or cosuppressors required for transcription, or the binding to its ligand, all-*trans* RA, is weak. In this way, all-*trans* RA regulation of gene expression is impaired. This fact has been used to study the impact of basal all-*trans* RA on both development and lymphocyte differentiation and proliferation.^{26,25} Beside RAR single mutants, the double mutants RAR α /RXR α , RAR β /RXR α , and RAR γ /RXR α have been created and exhibit profound developmental defects.²⁷ These mice exhibit severely reduced viability and numerous congenic abnormalities that do not appear in VAD.²¹ Together, these observations show that all-*trans* RA is the absent metabolite responsible for the defects observed under vitamin A deprivation and that all-*trans* RA plays an essential role in development through RARs.

With respect to RXRs, deletion of RXR α results in early lethality.²⁸ Further insight into the function of RXR α came from a study in which N-ethyl-N-nitrosourea (ENU) random germline mutagenized mice developed a hypomorphic allele of RXR α .²⁹ This strain of mouse, termed the “pinkie” mouse, exhibited an RXR α receptor with an altered ligand binding and heterodimerization domain that was poorly responsive to all-*trans* RA.²⁹ The characterization of this mutant mouse revealed widespread defects in the immune system, as will be discussed later. The generation of a floxed RXR α mouse has allowed for the tissue- and cell-type-restricted deletion of this receptor, and further implicated RXR α in immune function.³⁰ Recent studies have identified RAR α as the receptor critical for Foxp3 upregulation in T^{reg} (discussed below).

The Effect of RA on the Immune System

Given the profound reduction in resistance to infection that vitamin A deficiency causes, many studies have tried to resolve the cellular and molecular basis for the immune deficiency imposed. The following section discusses our current knowledge of the role that RA plays in immune homeostasis.

Effect on Antigen Presenting Cells

Monocytes/Macrophages—RA has been evaluated as to its effects on the activation and function of monocytes/ macrophages and the ability of macrophages to initiate immune response against pathogens.³¹ In general, the effect of RA on monocytes, macrophages, and macrophage cell lines suggests that RA inhibits the production of cytokines that favor the generation of Th1-type T cells and enhances the production of cytokines favoring the Th2-type T cells.

Under activation conditions, macrophages produce inflammatory cytokines like tumor necrosis factor (TNF)³² and nitric oxide (NO), which is a highly reactive free radical molecule. Aggarwal and colleagues analyzed the effect of all-*trans* RA on activated peritoneal macrophages and observed that all-*trans* RA dramatically inhibited the mRNA levels of TNF and reduced NO production from LPS-activated macrophages.³³ Similarly, using cord blood mononuclear cells as well as the human monocyte cell line THP-1, it was observed that RA enhances the secretion of IL-10 and inhibits inflammatory cytokines like TNF and IL-12.³⁴ Kim and colleagues evaluated the effects of RA on mouse macrophages and its indirect effect on T cells.³⁵ In their experiments, macrophages were pretreated with RA and subsequently activated either with LPS or heat-killed *Listeria* (HKL). As shown in previous studies, RA inhibited the production of pro-inflammatory mediators (IL-12 secretion) by activated macrophages. The researchers also performed an *in vivo* experiment in which mice received i.p. injections of retinoids and after 24 h splenic macrophages were isolated. LPS-or HKL-activated macrophages from retinoid-treated mice also secreted less IL-12 than did PBS-treated mice. Finally, to investigate if these RA-modulated macrophages may have an impact on the balance of Th1/Th2 type T cells, RA-treated macrophages were incubated with *in vivo* HKL-primed CD4+ T cells. Again, IL-12 levels were reduced when RA-treated macrophages were used as antigen presenting cells (APCs) in cocultures, and more importantly, T cell-derived IFN- and IL-4 levels were downregulated and upregulated, respectively. These results show that RA promotes the generation of Th2-skewed T cells through its impact on macrophages.³⁶ In conclusion, RA modulates the response of macrophages under activation conditions (inhibition of Th1 inflammatory cytokines), which will provide a microenvironment to allow or enhance the generation of Th2 type cells when the Ag is encountered. How RA “programs” APCs phenotype and function is still unknown, and whether RA influences tissue homing of macrophages is yet to be resolved.

Dendritic Cells—Dendritic cells (DCs) are the primary sentinel cells for triggering the development of adaptive immunity. These cells are able to act as APCs and initiate immune responses by providing antigen and costimulatory signals to T cells.³⁷ Thus, any effect that RA may exert on this cell type could have profound implications for the development of adaptive immunity. The functional impact of many retinoids (retinol, 9-*cis*, and all-*trans*-RA) on human monocyte-derived DCs (MoDCs) has been evaluated.³⁸ Investigators observed that retinoids induce apoptosis in immature but not mature MoDCs, which was inhibited by the secretion of inflammatory cytokines like TNF and IL-1. In addition, they demonstrated that retinoids, together with inflammatory cytokines (but not with retinoids alone), enhanced the upregulation of MHC-II and CD86 expression on MoDCs. The up-regulated expression of these molecules may explain in part, the enhanced allogeneic T cell proliferation seen when retinoid-treated MoDCs were used in the cocultures. In parallel, retinoids cooperated with inflammatory signals (cytokines and CD40 signaling) to improve the ability of MoDCs to present antigen. Using specific synthetic agonists and antagonists, it was shown that RA modulated the phenotype and function of immature MoDCs via RAR / RXR signaling, indicating a direct effect of RA through its pathway on DCs.³⁸

RA and GM-CSF enhanced the differentiation of monocytes into dendritic-like cells. These RA-DCs exhibited DC morphology and had the phenotype of immature DCs, with increased expression of CD1 a, adhesion, and costimulatory molecules. RA-DCs were more effective at inducing CD4⁺ T cell proliferative responses and increased IL12 production and, unlike macrophages, drove T cells toward an IL12-dependent T-helper cell type 1 response with secretion of IFN γ .³⁹

In another study, porcine MoDCs were used to evaluate the role of RA and its ability to induce gut-homing markers on T cells.⁴⁰ A published study reported that a tissue-resident DC will instruct or “imprint” a T cell to migrate to that tissue by inducing the expression of homing markers (α 4 7 and CCR9 for gut-homing mediated by RA-producing DC, or P- and E-selectin ligands for skin-homing, for example).^{41,42} Based on these observations, MoDCs were treated with RA and analyzed for the expression of α 4 7 and mRNA levels of CCR9 on T cells.⁴⁰ The researchers found that RA induces these gut-homing markers through modulation of MoDC phenotype and function. Also, they observed that RA-treated MoDCs were more effective at inducing T cell proliferation, as noted above. However, when the phenotype of the treated MoDCs was evaluated, no differences in the expression of MHC-II or CD80/86 molecules were found, even though the capacity to endocytose and process Ag was clearly reduced. To assay whether soluble factors were the players in imprinting, transwells experiments were performed, which demonstrated that soluble molecules, not cell contact, were necessary. TGF- β and IL-6 production by RA-DCs were shown to contribute, at least in part, to T cell imprinting.

Taraboletti and colleagues⁴³ explored the impact of RA in murine bone marrow-derived DC (BMDC) to clarify the mechanism by which RA induces the differentiation and migration of DCs in RA-treated leukemia cells. In their study, they observed that when RA-treated BMDC were injected intratumorally into tumor-bearing mice, the RA-treated BMDC had a demonstrably heightened capacity to migrate to the regional lymph node (LN) compared with untreated BMDC. This result suggests that RA affects BMDC migratory properties, a conclusion that was confirmed when they analyzed the mRNA expression and secretion of matrix metalloproteinases (MMPs). MMP-9 and -14 were highly increased under RA exposure, while tissue inhibitors of MMP-1, -2, and -3 (TIMPs) were downregulated.⁴⁴ These observations suggest that RA induces migration of DC from the tumor to draining lymph nodes through the secretion of molecules that permit the exit from the tumor microenvironment/matrix, allowing the DC to migrate to the appropriate place to encounter T cells and present Ag. In this way, RA may be able to enhance the initiation of immune responses against tumors.

Immature myeloid-suppressive cells (ImC) are another class of APCs that can exert profound effects on the development of acquired immunity. Heightened ImC infiltration has been found in a wide spectrum of tumors, and it is believed that they contribute to the impairment of immunity typically seen in.⁴⁵ In a clinical trial, patients with metastatic kidney cancer were orally treated with different concentrations of all-*trans* RA. The patients treated with the high dose of RA showed no change in number of white blood cells, neutrophils, or lymphocytes, but the number of ImCs was significantly reduced. When total populations of DC were analyzed, no drastic changes were observed, but when subsets were evaluated, RA treatment decreased the proportions of myeloid DC (previously described as DC supporting the tolerogenic microenvironment under tumor conditions) and did not alter the fraction of plasmacytoid DC. In addition, DCs from RA-treated patients revealed a more potent stimulatory effect on T cells when tested in a mixed leukocyte reaction (MLR) in comparison with DCs from untreated cancer patients. This observation is consistent with findings of the previous *in vitro* studies on DCs that are cited above. Also, T cell production of IFN- γ (Th1 cytokine) and IL-2 was increased, while the Th2 cytokine IL-5 was reduced,

demonstrating that RA administration affects DC function to promote a Th1 response in cancer patients.⁴⁶ These important observations reveal a valuable immunoregulatory property of RA as a pharmacological agent for treatment of cancer, where RA may be able to boost the development of antitumor immunity. At odds with these observations in humans are a plethora of murine studies that suggest that RA tends to drive immune responses in the Th2 direction. Future studies must come to terms with this conundrum. The complexities of the systems are significant. Studies must now carefully discriminate the functions of discrete RARs and/or RXRs in regulating defined immunological events in defined myeloid and dendritic cells.

B Cells—B cells are the antibody-producing cells of the immune system and are also important as APCs that can capture antigen through their antigen-specific receptor and trigger the development of class I and class II restricted T cell responses. B cells, as central elements in adaptive immunity, have also been studied to determine how RA may influence their function. Smith and Hayes evaluated Ab response against two protein Ags in vitamin A-deficient mice.² Their study found IgG1 synthesis to be drastically impaired, while IgM production was unaffected. It appeared that the impact on Ig isotype switching was due to an effect on T cells since CD4⁺ T helper cells from vitamin A-deficient mice were unable to induce IgG secretion by either sufficient or deficient vitamin A B cells.⁴⁷ They also showed that macrophages and B cells from vitamin A deficient mice showed normal function. The impaired B cell responses in the absence of vitamin A no doubt significantly contributes to the increased susceptibility of vitamin A deficient individuals to infectious diseases.

Using an alternative approach, the direct effect of exogenous RA on both human cord blood mononuclear cells (CBMC) and adult peripheral blood mononuclear cells (PBMC) was studied.⁴⁸ Investigators observed that activated CBMC produced higher levels of IgM when activated in the presence of RA. RA seemed to promote B cell differentiation into Ab-secreting cells. When adult cells were tested, RA enhanced IgG production by CBMCs. To answer whether RA was affecting Ab production by modulating B or T cell function, purified T cells were incubated with RA and the supernatant was used in the culture with B cells. This approach showed that RA affected T cell function, possibly by modulating the release of some unidentified soluble factor(s). In addition, RA was shown to induce the overproduction of IL-6 by activated B cells.⁴⁸ The direct impact of RA was subsequently confirmed when it was demonstrated that the addition of exogenous RA to human B cell cultures enhanced the terminal differentiation of B cells to plasma/Ab-producing cells. This effect of RA was CD40 signaling-dependent.⁴⁹ Hence, RA can exert either direct or indirect effects on B cell isotype switching and antibody production.

Effect on T Lymphocytes

It has been known for over 75 years that vitamin A as a dietary supplement enhances host resistance to infectious disease (as reviewed by Fawzi and Villamor⁵⁰). Of course, T cell function, as well as B cell function is critical to maintain host resistance. A direct impact of RA on peripheral T cell numbers has been demonstrated in humans, where vitamin A supplementation in areas of malnutrition has been shown to elevate numbers of peripheral CD4⁺ T cells in children. Vitamin A could influence both T cell ontogeny and the function and differentiation of mature T cells.

T Cell Ontogeny—RA may exert its impact on the development and selection of the T cell repertoire. RA promotes cell proliferation via the inhibition of apoptosis.⁵¹ Zsondy and colleagues exploited this observation to ask if RA could influence T cell negative selection in the thymus. Using an *in vivo* model, they stimulated T cells either by anti-CD3 antibody or by Ag, and showed that these stimuli induced apoptosis of the immature population of T

cells [CD4⁺CD8⁺ thymocytes (DP)]. Apoptosis was correlated with the upregulation of the orphan receptor *nur77*. The use of an RAR agonist blocked apoptosis but did not reduce either the mRNA expression or the protein levels of *nur77*. Because *nur77* binds to DNA to exert its function, the group performed DNA binding experiments and found that the RAR agonist interfered with DNA binding ability of *nur77*, therefore modulating the gene-targeting activity of *nur77*. These results showed that retinoids may play a role in the development of T cells in the thymus, specifically by preventing negative selection.

A recent report focused on the analysis of RAR/RXR expression in thymi of young children (<5 years old). This study revealed a pattern of expression depending on the age of the individuals: RAR and RXR are highly expressed in the first year after birth, and the RAR is expressed at later ages. They also showed that the immature DP is highly represented in the early stage of thymocyte cultures, but is reduced over time, together with the emergence of mature CD8⁺ T cells. The addition of all-*trans* RA to the culture enhanced the DP population and decreased the percentage of mature CD8⁺ T cells; however, there was an overall positive effect on the number of mature CD4⁺ T cells in the presence of RA. The effect of all-*trans* RA was accompanied by an augmentation in the expression of RAR mRNA levels.⁵² Therefore, RA may augment total peripheral T cell numbers by exerting its effects centrally within the thymus by enhancing the egress of mature T cells. More recently,⁵³ an age-dependent change has been shown in mRNA expression of retinaldehyde dehydrogenases (RALDH1/2), cellular RA binding protein-II, and CYP26 A in the postnatally developing thymus. While these investigators could not clearly detect RA in thymic homogenates, they could measure the RALDH1-dependent induction of a RAR-responsive transgene. RALDH1 was located in thymic epithelial cells. Thus, these data provide evidence for endogenous retinoid synthesis in the thymus and suggest that retinoids similar to glucocorticoids might indeed be involved in the regulation of thymic proliferation and selection processes.

Mature T Cell Subsets—Little more is known about the effect of RA on T cell selection; however, vitamin A-deficient models have shed light on the impact of vitamin A deficiency on mature T cell populations. Smith and Hayes have developed a system to create vitamin A-deficient mice.⁵⁴ Using this approach, they showed that both delayed type hypersensitivity responses and antibody responses in these mice were impaired, both of which have been the result of a diminished T helper population.^{47,55} In addition, they observed that T cells from vitamin A-deficient mice produced higher levels of IFN- γ , which may reduce the generation of Th2-type T cells. APCs from the deficient mice induced T cells to secrete heightened levels of IFN- γ , and supplementation with RA inhibited the activity of these APCs, concordant with some *in vitro* studies. Using the vitamin A-deficient model, these investigators described three major roles for RA in regulating cell-mediated immunity: downregulation in the activities of Th1 type T cells (IFN- γ secretion), reduced APC function, and enhanced Th2 type T cell growth and/or differentiation.⁵⁶

These findings led others to more incisively evaluate the impact of RA on the balance of Th1 and Th2 type T cells. In this context, the impact of RA on DP thymocytes activated *in vitro* in the presence of Th1 (IL-2 plus IL-12) or Th2 (IL-2 plus IL-4) type conditions was evaluated. The addition of RA to Th1 polarizing condition blocked the generation of Th1-type T cell, yet it enhanced the generation of Th2 type T cells in Th2 polarizing condition. This is consistent with the studies showing that vitamin A deficiency enhanced IFN γ production by T cells. The analysis of transcription factor expression [either Th1 (T-bet and IL-12R β 2) or Th2 response (c-maf, IL-4R α , and GATA-3)] was consistent with the impact on cytokine production by RA.^{57,58} The use of RAR-specific agonists and antagonists identified RAR α and RAR β as the players in the promotion of Th2 T cells.

In addition to its impact on Th1/Th2 balance, RA has been shown to exert profound effects on another mature T cell subset, Th-17 T cells. Th-17 cells are characterized by the secretion of the cytokine IL-17 and are associated with the pathogenesis of autoimmune diseases.⁵⁹ Th-17 cells are induced in the presence of TGF- β and pro-inflammatory cytokines like IL-6, IL-1, or TNF- α .⁶⁰ It has been shown that the orphan nuclear receptor ROR γ t is necessary for Th-17 cell differentiation.⁶¹ Mucida and colleagues evaluated the role of RA in the generation of Th-17 cells *in vitro* and *in vivo*, and showed that RA inhibited the differentiation of Th-17 cell *in vitro*. RA induced a profound reduction in the expression of ROR γ t in T cells cultured under Th-17 conditions, resulting in a blockade in Th-17 differentiation. The negative effect of RA on the generation of Th-17 cells was also established *in vivo*⁶² in mice lacking the ROR γ t receptor. In summary, it appears that at multiple levels, RA can facilitate the skewing of T cell responses away from an inflammatory Th1/Th17 phenotype and towards a Th2-polarized response.

T Cell Homing—In addition to its impact on T cell skewing, RA affects the homing behavior of T cells. The peripheral homing “preferences” of T cells to migrate to a specific tissue are imprinted by DCs from that tissue when Ag is presented.⁴² In the cited study, the investigators showed that DCs from the gut—mesenteric lymph nodes (MLN) and Peyer’s patches (PP)—induce the gut-homing markers α 4 β 7 and CCR9 on T cells, while DCs from peripheral lymph nodes (PLN) induce, preferentially, the skin homing receptors P- and E-selectin ligands. Based on this observation and the fact that vitamin A deficiency impairs gut immunity, Iwata and colleagues⁴¹ evaluated the function of RA in the expression of homing receptors on T cells. In their experiments, CD4⁺ T cells were stimulated polyclonally with anti-CD3 and anti-CD28 in the presence or absence of RA. The addition of RA enhanced the expression of α 4 β 7 and the mRNA levels of CCR9, but suppressed the expression of E-selectin ligand. This effect was also observed under Th1 or Th2 skewing conditions. In addition, CD4⁺ T cells cultured in the presence of RA showed increased migration to the CCR9 ligand TECK when tested in trans-well experiments. Performing competitive homing experiments *in vivo*, the group also observed that RA-treated CD4⁺ T cells migrated preferentially to the gut, in comparison to untreated CD4⁺ T cells. The investigators also identified the potential population of cells synthesizing RA *in vivo*. For this purpose, they first evaluated the expression of the enzyme RALDH and found that MLN- and PP-DCs, but not PLN-DCs, express RALDH. In addition, they analyzed the capacity of DCs isolated from different organs to metabolize retinol to RA during Ag presentation *in vitro*. MLN- and PP-DC were able to convert retinol to RA, but splenic DCs were unable.⁴¹ This work was the first to demonstrate that the migration of T cells to the gut is triggered by the production of RA by gut-resident DCs and provided at least one explanation for the pivotal role of RA in controlling the development of gut immunity. Finally, the observation that MLN- and PP-DCs, but not splenic DCs produced RA explains why MLN- and PP-DCs are unable to induce the differentiation of Th-17 cells *in vitro*.⁶² The production of RA by different DC populations is no doubt important, as RA controls homing and phenotype of the T cells that engaged by the DC.

In addition to this study, Mora and colleagues⁶³ studied the role of DCs and RA in the induction of gut homing on B cells, replicating the observations previously made on T cell populations. In their work, gut-resident DCs induced α 4 β 7 and CCR9 gut-homing markers on B cells, which was dependent on RA. They also demonstrated the ability of gut-resident DC-cultured B cells to migrate preferentially to the gut *in vivo*. When the IgA production by B cells was analyzed, RA induced IgA at higher levels only when gut resident DCs were present. RA showed a synergistic activity in IgA induction together with IL-6 and IL-5. Finally, the impact of RA in inducing gut homing markers has been reproduced using human DCs and B cells.⁶³

RA, Regulatory T Cells (nT^{reg}), and Tolerance

Natural CD4⁺ CD25⁺ T^{reg} (nT^{reg})—nT^{reg} represent 5–10% of peripheral CD4⁺ T cells in naive mice and humans. It is believed that these cells emerge from the thymus as a consequence of high-affinity interactions with self-antigen, avoiding negative selection and escaping to the periphery.⁶⁴ The functional importance of this subset of cells emerged from the early studies of Sakaguchi and colleagues who demonstrated the critical role of these cells in maintaining peripheral tolerance.^{65–73} Later studies by Wood^{74,75} and Waldmann^{76–79} in transplantation systems and Shevach in autoimmunity and infectious disease⁸⁰ demonstrated the important regulatory function of these cells *in vivo*. The foxhead box P3 transcription factor (Foxp3) is a specific molecular marker for the nT^{reg}.^{81–84} Recently, both Rudensky⁸⁵ and Flavell⁸⁶ reported the creation of mice that report a fluorochrome when Foxp3 is transcriptionally activated. Use of these Foxp3^{GFP/RFP} mice⁸⁵ has greatly facilitated the identification of T^{reg} *in vivo*. As with other T and B cell subsets, RA can affect the homing of nT^{reg} as it has been showed to induce the gut-homing molecule a4b7. The a4b7⁺ T^{reg} were suppressive *in vitro* and migrated to the gut under inflammatory conditions. Other effects of RA will be discussed later in this review.

Inducible/Adaptive (a)T^{reg}—In contrast to nT^{reg}, RA exerts a tremendous impact on the differentiation of aT^{reg}. aT^{reg} arise in the periphery from mature CD4⁺CD25⁻ T cells given particular cytokine environments (notably TGFb), by presentation by immature DCs, or specific DC subsets (like MLN-DC), or via particular routes of antigen administration (such as nasal routes⁸⁷). For practical purposes aT^{reg} can be produced by the culture of CD4⁺CD25⁻ T cells *in vitro* with TGFb^{88,89} or through selective means of (under optimized) antigen presentation *in vitro*^{92–94} or *in vivo*.⁹⁵

Our data, and those of others,^{62,96–99} show that RA dramatically enhances the expression of Foxp3 by CD4⁺ T cells and greatly enhances the expansion and function of aT^{reg}. Because RA induces gut homing, most studies have focused on the role of RA in regulating gut immunity via the induction of T^{reg}, however, we propose that RA plays a more general role in regulating peripheral tolerance.

Studies from our lab⁹⁶ demonstrated that RA greatly enhanced the expression of Foxp3 in CD4⁺ T cells stimulated with antigen or anti-CD3 and TGFb. The results were quite striking, in that almost 100% of T cells were Foxp3⁺ in the presence of RA. Even more important is that RA enhanced the growth of the Foxp3⁺ T cells, increased their suppressor activity, and made them resistant to reversion to a Foxp3⁻ phenotype *in vivo*. In this sense, we propose that RA induces the terminal differentiation of T^{reg} to effector T^{reg}. One last important aspect of RA influence is that RA extinguishes the negative impact of costimulation on Foxp3 expression. That is, in cultures where one uses DCs as an APC source (or CD28 as a model of high costimulation), T cells activated under these conditions (in the presence of TGFb) do not become Foxp3⁺. However, the inclusion of RA allows for robust expansion and high levels of Foxp3 expression.

Studies by three other groups also showed a critical role for RA in T^{reg} development, but focused exclusively on its role in the gut (because of the ability of RA to induce gut homing) and also highlighted the negative impact of RA on the development of Th17. Mucida and colleagues⁶² showed that DCs derived from the MLN, but not DCs from spleen, induced Foxp3 expression in T cells. Second, they showed that MLN-DCs synthesized RA. Third, they demonstrated that the *in vivo* administration of an RA antagonist impaired the development of Foxp3⁺ T cells in the gut mucosa. These important findings established that RA antagonists, or metabolites of vitamin A, play a pivotal role in T^{reg} development *in vivo*. Another important aspect of the work from Mucida and associates, was that they showed that RA impeded the development of Th17 at the cost of enhancing the development of T^{reg}.

In addition to this study, Belkaid and colleagues⁹⁹ reported that naïve CD4⁺, Foxp3⁻ T cells converted to CD4⁺FoxP3⁺ T cells when they migrated to the gut. They identified that gut-resident DCs mediated this conversion of T^{reg} in a TGFb-and RA-dependent fashion. In a similar study, Powrie and colleagues⁹⁷ showed that conversion from naïve CD4⁺ T cells to T^{reg} occurs after oral administration of Ag. They also identified CD103⁺ gut-resident DC as the inducers of T^{reg} and, like all other studies, confirmed RA dependence. Taken together, these studies established a novel role of RA within the gut as a critical factor in inducing T^{reg} and maintaining gut immune homeostasis.

RA and Infectious Diseases

Vitamin deficiency and pathology were first correlated by the ancient Egyptians, who applied liver (known now as the storage for vitamin A) extracts to the eyes of people affected with nutritional night blindness.¹⁰⁰ It was not until the 18th century, in Europe, that scientists presented “nutritional theories” and advised the public on the advantages of breastfeeding and intake of fat (See review by Semba,¹⁰¹ ben). In 1892, it was suggested that diet could have an impact on susceptibility to infectious diseases based on the observation that children suffering measles or whooping cough also developed xerophthalmia (blindness produced by vitamin A deficiency). Later studies indicated that supplements with carrots could reduce the number and severity of respiratory infections.¹⁰⁰

In the following years, laboratory studies (using animal models) focused on unraveling the components of food that affected human health and disease. Osborne & Mendel (United States), Hopkins (England) and Pekelkaring (Germany), in parallel, concluded that a factor “essential for life” is contained in butterfat. Casimir Funk set out to purify this factor, and the fraction he obtained he named “vital amina.” In 1913, McCollum & Davis reconfirmed the presence of this factor in butter and they renamed it “fat-soluble A,” which was ultimately permuted to the term “vitamin A.”¹⁰¹ Semba’s review describes historically how vitamin A became an “anti-infective” agent because of the diligence and insights of Edward Mellanby and his studies in vitamin A-deficient animals. These animals presented higher incidence of respiratory diseases and death than those on normal diets.

Continued use of animal models for the study of vitamin A in disease susceptibility and pathologies was instrumental in establishing a causal link between vitamin A deficiency and disease vulnerability. Vitamin A-deficient chickens infected with Newcastle virus (a system to mimic measles in humans) were shown to have a decrease in T cell number in the primary lymphoid organs together with lower body weights when compared to a control group.¹⁰² Due to the high prevalence of vitamin A deficiency in children and exposure to malaria in India, Krishna and colleagues¹⁰³ used a rat model to study the immunological mechanism underlying this infection. They took advantage of the parasite *Plasmodium berghei*, which in rats is normally a self-limiting infection. However, vitamin A-deficient rats that were parasite infected experienced an increase in parasitemia and death rate compared to controls. Much emphasis in vitamin A deficiency has focused on macrophage and/or APC impairment. When “glass adherent cell” function was studied, the investigators concluded that the cells from a control group could eliminate most of the parasite compared to cells from rats on a vitamin A-deficient diet.¹⁰³ Years later, others demonstrated that peritoneal macrophages from vitamin A-deficient chickens infected with Newcastle virus were impaired in their phagocytic and killing activities. This was believed to inevitably contribute to the heightened development and progression of disease in the vitamin A-deficient chickens.¹⁰⁴ In a model of *Staphylococcus aureus*-induced arthritis, Wiedermann and colleagues observed that the weight of vitamin A-deficient rats was four times lower than that of control animals, that arthritis was more severe, and that pathological kidney lesions and accumulation of *S. aureus* in the joints was more profound in vitamin A-deficient animals, as was the T cell proliferative. Interestingly, as with previous reports, both

phagocytic activity and intracellular killing by peritoneal macrophages from vitamin A-deficient rats were reduced compared to animals on control diets. Finally, sera from infected vitamin A-deficient rats had a 50% reduction in hemolytic activity compared with sera from infected but sufficient rats.¹⁰⁵ Together, morbidity, mortality, and frequency and severity of arthritis were enhanced under vitamin A deficiency, probably due to chronic hyper-reactive T cells resulting from the inability of the host to eliminate the pathogen. While it was not explicitly evaluated, one might suggest that the vitamin A deficiency impaired the clearance of the pathogen, resulting in enhanced, persistent Th1 skewing, with exacerbated arthritis.

Similar to the observation in models of bacterial infection, Nauss and colleagues¹⁰⁶ showed that vitamin A status could influence the outcome of herpes simplex virus (HSV) infection in the eye of rats. Vitamin A deficiency resulted in rats presenting with more severe lesions and higher levels of inflammation, cellular infiltration, and edema. However, in this report, no differences in a variety of immunological parameters (including cell number and antibody titer) when compared with the control group were elucidated.

More recent human studies have shown that deficiency of vitamin A is associated with heightened incidence of infectious diseases, including diarrheal and respiratory diseases, malaria, tuberculosis, leprosy, rheumatic fever, otitis media, HIV-1, and others. A clinical trial involving children from Indonesia studied vitamin A supplementation and showed an increase in the levels of IgG in response to tetanus toxoid. Further studies showed that high dose of vitamin A supplementation in children with measles increased the number of circulating T cells, and also that vitamin A supplementation could reduce the incidence of respiratory infections in children.³¹ For a more detailed description of the clinical trials involving vitamin A supplementation, we suggest Villamor's review.⁵⁰

Autoimmunity

The breakdown in self-tolerance of the immune system and the emergence of self-reactive T and B cells result in the development of chronic inflammatory responses directed against the host. Autoimmune diseases are those characterized by the presence of auto-antibodies and/or autoreactive T cells, which result in systemic or organ- or tissue-specific inflammation. Examples of autoimmune diseases are systemic lupus erythematosus, rheumatoid arthritis, sclerosis multiple, and Crohn's disease.

As discussed in this review, RA can exert a Th2 skewing on immunity. In addition it has been shown to reduce the infiltration of leukocytes into sites of inflammation and reduce the production of pro-inflammatory cytokines both *in vivo* and *in vitro*. Some of these effects may be by RA enhancement of TGF β production.¹⁰⁷⁻¹⁰⁹

In view of the fact that retinoids can, in general, exert immunosuppressive effects, they have been considered as single or adjuvant agents to treat autoimmune diseases. Multiple sclerosis (MS) is characterized by an inflammatory attack on the central nervous system resulting in demyelination and progressive neurological deficits. This pathology is mimicked in a murine model (experimental allergic encephalomyelitis, EAE) in which myelin basic protein (MBP)-specific CD4⁺ T cells are transferred to animals and signs of the diseases can be measured. Using this approach, Racke and colleagues studied the use of retinoids to treat EAE.¹¹⁰ These studies showed that RA inhibited the proliferation of MBP-specific T cells and delayed the onset of the disease. Also, the study demonstrated that RA enhanced mRNA for IL-4 and reduced mRNA for IL-2, IFN γ , and TNF α , suggesting that RA promotes a Th2 T cell phenotype. Similar findings were observed using human MBP-specific T cells, in which RA diminished the proliferation capacity of the human MBP-specific T cells in a dose-dependent manner and enhanced IL-4 synthesis.¹¹¹ When the retinoid, 4-

hydroxyphenylretinamide (4-HPR) was used as a therapeutic agent in EAE, stabilization of the disease was observed.

Given the success of retinoic monotherapy, Qu and colleagues¹¹² asked whether retinoids, in the context of standard of care drugs like IFN γ could exert an improved therapeutic benefit. Their work showed that RA enhances IFN γ -1b activity in augmenting CD8⁺ suppressor cell function *in vitro*, and RA was able to inhibit the production of IFN γ secreting cells, which has been promoted by IFN γ -1b administration. However, no meaningful change was noted in disability or quality of life over the course of the Phase I clinical trial. In those who completed the trial, neuropsychological testing indicated improvement on selected aspects of verbal memory at 6 months compared with baseline values. Focusing on the role of microglia and astrocytes in driving inflammation under pathological states, Xu and colleagues¹¹³ analyzed the impact of RA on these cell types. They observed that 9-*cis*-RA inhibits the pro-inflammatory factors NO and TNF α secreted by murine LPS-treated microglia and astrocytes, suggesting that under inflammatory conditions (disease) the administration of this retinoid may decrease inflammation. Taken together, the general immunosuppressive effects of retinoids seemed to translate to autoimmune indications.

Systemic lupus erythematosus (SLE) is another autoimmune disease in which the possible therapeutic value of retinoids has been studied. In the 1980s, Gershwin and colleagues observed that zinc-deficient diet decreased the production of autoantibodies and prolonged the survival of lupus-prone strains of mice (NZB, NZB/W, and MRL/1). They hypothesized that zinc deficiency may perturb vitamin A metabolism. Using a murine model for lupus, they analyzed dietary vitamin A intake and its relation with the outcome of disease. Their work showed that NZB mice manifested more severe signs of the disease (enhanced hypergammaglobulinemia and autoantibody production) when vitamin A deficient.¹¹⁴ Utilizing another approach, Kinoshita and colleagues¹¹⁵ administered RA to SLE-prone NZB/W mice and showed that RA promoted survival, reduced proteinuria, suppressed kidney-infiltrating cells (T and B cells and macrophages), improved overall renal structure, inhibited kidney deposits of IgGs, reduced splenomegaly and production of autoantibodies, and diminished IL-10, IFN γ , and IL-2 mRNA levels. The same observations were replicated using other murine models for SLE (MRL/lpr mice).¹¹⁶

Retinoids have also been shown effective in the management of other autoimmune disease models. Studies in the DBA/1 J model of collagen-induced arthritis, demonstrated that three weekly administrations of ATRA reduced overall disease. In this study, the arthritis score and incidence in mice were lowered, the histological scores in the disease were reduced by 34%, and there was lowered macrophage infiltration into the joints. Furthermore, ATRA treatment lowered the production of pro-inflammatory cytokines and anticollagen antibody.¹¹⁷ In a TNBS-induced colitis model, RA has been shown to ameliorate disease. RA, administered daily after disease induction, reduced TNBS-induced fibrosis.¹¹⁸ While we do not know the cellular and molecular mechanisms that are important in impairing autoimmunity, the ability of RA to induce aT^{reg} and alter leukocyte homing are likely candidates in its action.

Conclusion

The last five years have witnessed a revolutionary change in our understanding of how vitamins control immunity. The impact of vitamin A and vitamin D metabolites on leukocyte homing and the conceptual development of “imprinting” as a means to anatomically orchestrate the immune response represent major advances in immunology. Only in the last year, we have come to appreciate that RA also controls the differentiation of CD4⁺ effector T cells to CD4⁺, Foxp3⁺ regulatory T cells. Taking advantage of these novel

mechanisms of action of RA, new strategies can now be formulated to use RA agonists and antagonists to control inflammation via control of homing and T^{reg} differentiation. Furthermore, we have yet to fully explore the role of the various RAR and RXR receptors in each of these novel immunological mechanisms. Once we link specific receptors to defined immunological functions, specific RAR and/or RXR agonists and antagonists can be used to fine tune the impact on the immune system and, hopefully, immune-related diseases.

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