BioFactors

Retinoic acid: A key player in immunity

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Abstract.

For the past 100 years, vitamin A has been implicated as an essential dietary component in host resistance to infectious disease. However, only recently have studies begun to elucidate the cellular and molecular mechanisms of how vitamin A regulates cell-mediated and humoral-mediated immunity. In this review, we present an overview of the recent discoveries of the role that vitamin A and its metabolite, retinoic acid (RA), play in the regulation of immune cells. How RA impacts on leukocyte growth, differentiation, and homing is discussed with special attention to inflammatory responses and solid tumor microenvironment.

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1. Introduction

Vitamin A from the diet provides the retinoids necessary for a variety of biological functions including embryonic development, vision, brain function, and many others. Retinol, the alcohol form of vitamin A, gives rise to the acid form retinoic acid (RA), which is the metabolically active form of vitamin A. All-*trans*- and 9-*cis*-RA are the potent regulators of gene expression and play an essential role in the modulation of cell proliferation and differentiation [1]. For more than four decades, the impact of vitamin A deficiency on immunity has been studied. These studies have repeatedly demonstrated the indispensable requirement of this natural product to maintain host defense to bacterial, viral, and protozoal diseases [2,3]. The findings for a role of vitamin A in the regulation of inflammatory T cells and adaptive regulatory T cells (aT^{reg}) differentiation represent a significant advance in our understanding of the relationship of vitamin A and immunity. The current understanding as to how vitamin A impacts on the development of immune cells is discussed.

2. RA mediators

2.1. Enzymes

Vitamin A needs to be catabolized to different metabolites to exert its function. To do this, a group of enzymes, divided in three families, will act together to form the final compound RA. The first step is the conversion of vitamin A to retinal and is catalyzed by the alcohol dehydrogenase (ADH) family. ADHs have specificities for ethanol, retinoids, and other alcohols and aldehydes of physiological importance [4]. This step can also be regulated by the short-chain dehydrogenase/reductase family, which shows a wide affinity for alcohols and aldehydes [5]. Additionally, the aldehyde dehydrogenase (ALDH, also known as RALDH) family participates in the conversion of aldehydes to carboxylic acid compounds (acids) [6]. It has been shown that RALDH levels are regulated by vitamin A, such that in vitamin A-deficient animals, RALDH expression is greatly reduced [7]. As expression of RALDH has been found on immune cells, it is tempting to propose that RA may play a role in the development of immunity. One more critical element in RA biosynthesis is the major RA metabolizing enzyme, CYP26A, which has been very well established in developmental models to disrupt RA metabolism by promoting RA catabolism [8–10]. Little is known about how the expression of this enzyme may influence the development of immune responses.

2.2. Retinoid-binding proteins

Retinoids are not found free extracellularly; instead, they are associated with binding proteins for delivery to target tissue, usually over very short distances. The fact that RA acts only over a short distance and within a prescribed microenvironment is much like the activities of cytokines. Once in the cell, retinoids associate with cellular membranes or will be transported/stored coupled to intracellular proteins. There are three main families of retinoid-binding proteins: retinol-binding protein (RBP), cellular retinol (or

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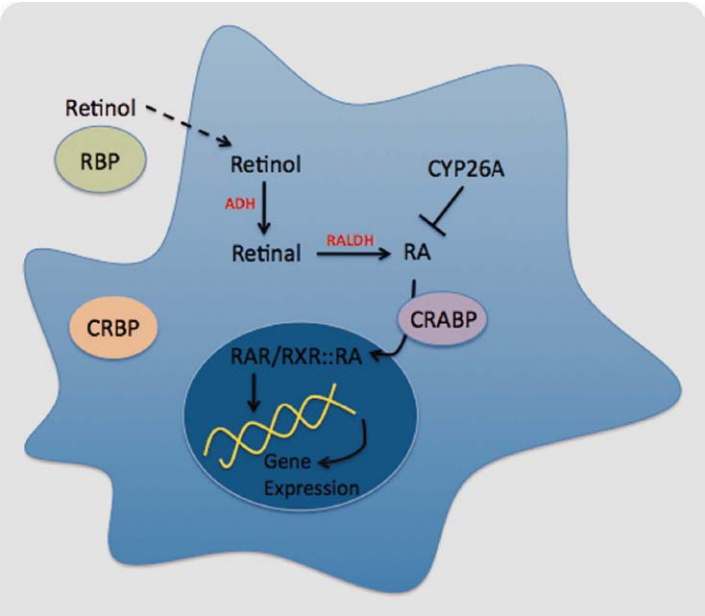

Fig. 1. RA metabolism. Retinol gives rise to RA through the activity of different families of enzymes, including RALDH, which catalyzes the last step in an irreversible manner. Once in the cytoplasm, RA binds to CRABP and is transferred to the nucleus, where it is recognized by the nuclear receptors (RAR/RXR), and it binds to the DNA to regulate gene expression. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

retinal)-binding protein, and cellular retinoic acid-binding protein (CRABP). RBP binds to retinol and its protein synthesis takes place not only in the liver but also in heart, testis, eyes, spleen, and others. Because the liver stores vitamin A, the expression and release of RBP-retinol will depend on the availability of vitamin A. It has been demonstrated that under vitamin A deficiency, RBP expression is downregulated and its release to circulation inhibited [11].

2.3. RA nuclear receptors

To regulate gene expression, all-*trans*- and 9-*cis*-RA bind to nuclear receptors, which act as ligand-induced transcription factors to bind to specific sequences in the DNA and modulate the transcription of target genes (Fig. 1). RA may bind to RA receptors (RARs) or to retinoic X receptors (RXRs), both of them belonging to the steroid/thyroid/vitamin D receptor super family.

RAR family contains three members: RARα (isoforms a1-2), RARβ (isoforms b1-4), and RARγ. The RXR family also contains three members (RXRα, RXRβ, and RXRγ) [12]. In attempts to dissect the role of RA signaling, genetically deficient mice were created in the early 90s. From these studies, it was shown that mice with deficiency in some of the receptor's isoforms appear normal (RARα1, RARβ, RARβ2, and RARγ2). However, null mutants in RARα [13] or RARγ [14] show some of the defects observed in postnatal vitamin A deficiency (pre and postnatal development malformations, male sterility, photoreceptor degeneration, and others). Hence, some of these mice are not suitable to study the role of different RAR/RXRs in immune regulation due to embryonic lethality or other severe phenotypes. To bypass the lethality issue of these null mutant strains, dominant-negative tissue-restricted RARα (dnRARα) have been created [15,16]. A dnRARα still binds to DNA and dimerizes with its partner, but either it cannot bind to coactivators/suppressors or the binding to its ligand (RA) is weak, as such alters RA signaling and the final regulation of gene expression. Using this system, dnRARα expression was directed to lung epithelia and it was shown that RA signaling through this receptor is required for normal alveolar development in neonatal lungs [16]. Utilizing the same approach, Mira-y-Lopez and coworkers demonstrated that mice expressing a dnRARα under the mouse mammary tumor virus promoter, which targets the expression of the transgene to some lymphocytes and mammary epithelia, developed B cell lymphoma [15]. This observation supports the physiological role of RA in the control of leukocyte differentiation and proliferation, which has been previously suggested by other groups [17,18].

With respect to RXRs, deletion of RXRα results in early lethality [19]. Stephensen et al. created a viable hypomorphic allele of RXRα induced by random mutagenesis. The characterization of this mutant mouse showed that the immune response is skewed toward a Th1-type response [19]. This observation supports previous reports where vitamin A was shown to enhance Th2 differentiation [20], but it carries the caveat that vitamin D, an additional RXRα ligand, can also alter the phenotype of T cells toward a Th2 phenotype [21]; therefore, the T-cell phenotype observed is not exclusively dependent on RA. Lloyd and coworkers took advantage of the Cre-Lox system to disrupt RXRα in the T-cell compartment by intercrossing lck-*cre* with the $RXR\alpha^{FLOX}$ mice. They observed a modest difference on B- and T-cell number, proliferation, cytokine production, and apoptosis *ex vivo* [22]. These studies suggest that the activation of RXRα signaling may contribute to T-cell differentiation, but the specific role of RA on this event needs to be specifically addressed.

3. The impact of RA on immunity

The recognition that poor nutrition positively correlated with an increased susceptibility to infectious disease dates back as far as the 18th century. The "nutritional theories" stated that milk and fat (source of vitamin A) are indispensable for the development of healthy children, and supplements of carrots could protect and ameliorate an infection. Studies on vitamin A supplementation indicated that children affected with xerophthalmia also suffered of respiratory diseases, wasting, and gastroenteritis, which were reduced when children received vitamin A supplements [23–25]. After the identification and purification of vitamin A (Frederick Hopkins, 1912), studies in which vitamin A was specifically eliminated in an experimental diet allowed scientists to assess the essentiality of this vitamin in immunity [26]. The study of host resistant to infectious disease in animals on vitamin A deficient diets began

to provide the first clues as to the role of vitamin A in immunity. We will briefly touch on the impact of vitamin A on a spectrum of hematopoietic cells but focus on the impact of vitamin A on the major cellular elements of the immune system.

3.1. Monocyte/macrophages

Overall, the impact 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 generation of Th2-type cells. A number of studies have shown that all-*trans*-RA modulates NO production, enhances interleukin (IL)-1 production, and inhibits the production of tumor necrosis factor (TNF)-α [27]. Kim et al. [28,29] evaluated the role of RA on mouse macrophages and the indirect effect on T cells. In their experiments, macrophages were pretreated with RA and subsequently activated with lipopolysaccharide (LPS). It was shown that RA inhibits IL-12 secretion by activated macrophages and these RA-treated macrophages when used as antigen presenting cells (APCs), reduced T-cell production of interferon (IFN)-γ and enhanced production of IL-4 were observed. Thus, overall RA signaling appears to establish a Th2-T^{reg} noninflammatory environment.

3.2. Dendritic cells

Darmanin et al. explored the impact of RA in murine bone marrow-derived dendritic cells (BMDC) with the purpose of clarifying the mechanism of how RA induces the differentiation and migration of dendritic cells (DCs) [30]. In their study, they observed that when RA-treated BMDC are injected intratumorally into tumor-bearing mice, these BMDC are found in draining lymph nodes (DLN), in comparison with the lack of migration injected with nontreated BMDC. This result suggests that RA is affecting BMDC migratory properties, which was confirmed when they analyzed the mRNA expression and secretion of matrix metalloproteinases (MMPs). MMP-9 and MMP-14 were highly increased under RA exposure, together with downregulation of tissue inhibitor of MMP-1, -2, and -3 (TIMPs). These observations suggest that RA induces migration of DC from the tumor to DLN through the secretion of molecules, which permit the exit from the tumor microenvironment/matrix, allowing the DC to migrate to the appropriate place to encounter T cells and present antigen (Ag).

Geissman et al. [31] assessed many retinoids (retinol, 9-*cis*- and all-*trans*-RA) and their effects on human monocyte-derived DCs (MoDCs). They demonstrated that retinoids, together with inflammatory cytokines (but not with retinoids alone), upregulate MHC-II, and CD86 expression on MoDCs supporting 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 Ag. Using specific synthetic agonist and antagonists, it was elucidated that RA modulated the phenotype and function of immature MoDCs via RARα/RXR signaling, indicating a direct effect of RA through its pathway on this APCs.

3.3. T lymphocytes

Much of what we know about the impact of vitamin A on T-cell immunity comes from studies in mice maintained on a vitamin A-deficient diet [2]. Overall, Hayes and coworkers showed that RA downregulated Th1 cells (IFN-γ secretion) decreased the activation of APCs and promoted Th2-cell growth and/or differentiation. Supporting the tenet that RA can bias immune responses to Th2-type responses, Iwata et al. [32] showed that RA impaired T cell skewing toward Th1 phenotype but allowed the development and expansion of Th2-type T cells. This observation was supported by the analysis of expression of transcription factors and molecules characteristic of either Th1 (T-bet and IL-12Rβ2) or Th2 response (c-maf, IL-4Rα, and GATA3). When RA was present in the cultures, in addition to inducing the secretion of Th2 cytokines, RA also promoted the expression of c-maf, IL-4Rα, and GATA3, together with reducing T-bet and IL-12Rβ2 [32]. The use of agonists and antagonists identified RARα and RARβ as the players in the promotion of Th2-type T cells.

3.4. Homing impact of RA

In 2004, Iwata et al. presented striking results involving RA as a key mediator in T-cell homing (or trafficking). In this study, they observed that RA "imprints" T-cell homing by inducing the expression of the gut-homing receptors $\alpha4\beta7$ and CCR9 on $CD4^+$ T lymphocytes. Most importantly, they showed that mainly gut-resident DCs (mesenteric lymph nodes and Peyer's patches derived) express the enzymes necessary to synthesize RA and also that on coculture with $CD4^+$ T cells, conversion of retinol to all-*trans*-RA takes place indicating that gut-DCs express metabolically active enzymes. It is important to note that splenic DC also display these properties but in a less extend [33]. Later, Svensson et al. also demonstrated that not only gut-resident DC but also splenic DC were capable of producing RA and inducing gut-homing receptors on T lymphocytes [34]. The homing properties of RA were also applicable to $CD4^+$ regulatory T lymphocytes (T^{reg}) and $CD8^+$ T and B lymphocytes [35,36]. These studies have made a major contribution to how nutrition may impact on immunity by elucidating how a dietary vitamin can dramatically alter gut immunity. These findings help to begin explaining how vitamin A deficiency may alter host resistance within the gut. Another significant advance in our understanding of the role of RA in immunity was the observation that RA enhanced the expression of the transcription factor FoxP3 on T^{regs} in a TGF-β-dependent fashion [37–39]. T^{regs} are one of the critical cellular subsets that control the development of inflammation and autoimmunity through suppression. The finding that RA exerts such profound effects on such important immunoregulatory subset has significant implications [40]. In addition to enhancing the differentiation of T^{reg}, it appears that RA also induces their irreversible commitment to the T^{reg} lineage. Hence, through its power to imprint T cells and enhance T^{reg} function and number, RA no doubt plays a critical role in maintaining the inflammatory/anti-inflammatory balance in the gut [16].

In 2005, Harrington et al. described a new population of T lymphocytes. These IL-17-producing $CD4^+$T lymphocytes (Th17 cells) showed inflammatory properties and the ability to mediate autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) and arthritis in mice [41]. Extensive characterization of Th17 cells also revealed that RA plays a role in Th17 generation. In this case, RA promotes Th17 differentiation at physiological concentrations (nM) but inhibits Th17 development at higher doses (42,43). Interestingly, RA besides imprinting gut-homing receptors on Th17 cells also seems to impact their activity as Th17 cells produced in the presence of RA showed enhanced inflammatory properties when assayed in a colitis mouse model [44]. These findings suggest that the levels of RA exquisitely control differentiation of different T-lymphocyte populations as it has been described for Th1, Th17, and $CD4^+FoxP3^+$ cells as well [45].

Besides Th1, Th2, and T^{reg} subsets, Th17 cells have also been described in response to allografts (transplantation) [46], tumors [47], and other conditions where inflammation takes place. These new insights into the proinflammatory and anti-inflammatory role that RA plays in controlling T-cell function provides a rationale basis for using RA agonists and antagonists for the management of immune-related diseases.

3.5. RA and the induction of aT^{reg}

Several populations of T^{reg} have been identified. Briefly, natural T^{reg} are developed in the thymus and express FoxP3 constitutively. On the other hand, T^{reg} can also be generated in the periphery, and they are denoted adaptive T^{reg} (aT^{reg}). In this regard, the presence of anti-inflammatory cytokines will determine aT^{reg} identity as TGF-β drives the induction of Th3 subset and IL-10 permits the development of Tr1 cells [48]. A number of studies [37–39,43,49] show that RA dramatically enhances the expression of Foxp3 by $CD4^+$ T cells and greatly enhances the expansion and function of aT^{reg}. Studies from our lab [39] demonstrated that RA greatly enhanced the expression of Foxp3 in $CD4^+$ T cells stimulated with Ag or αCD3 and TGF-β. The results were quite striking, in which 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 $Foxp3^-$ phenotype *in vivo*. 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 αCD28 as a model of high costimulation) T cells activated under these conditions (in the presence of TGF-β) do not become $Foxp3^+$. However, the inclusion of RA allows for expansion and high levels of Foxp3 expression.

Studies by three other groups also showed a critical role for RA in T^{reg} development, but these cases 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 et al. [43] showed that DCs derived from the mesenteric lymph node (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 a RA antagonist impaired the development of $Foxp3^+$ T cells in the gut mucosa. These important findings establish that RA, or metabolites of vitamin A, may play a pivotal role in T^{reg} development *in vivo*. Another important aspect of the work from Mucida et al. is 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, Sun et al. [37] reported that naïve $CD4^+$ Foxp3-T cells converted to $CD4^+FoxP3^+$T cells when migrated to the gut. They identified that gut-resident DCs mediated this conversion of T^{reg} in a TGF-β and RA-dependent fashion. In a very similar study, Coombes et al. [38] 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 confirmed the RA dependence. Taking together, these studies suggest that gut-derived RA may contribute to the generation and/or maintenance of Treg, which could be implicated in the control of inflammatory responses within this anatomical site.

3.6. RA in tumor immunity

RA may also play a role in regulating the immune response to tumors. Numerous studies have underscored the potent immunosuppressive impact of immature $CD11b^+Gr\text{-}1^+$ myeloid-derived suppressor cells (MDSCs) on the development of protective antitumor immunity in the host. Vitamin A-deficient mice [50] and pan-RAR antagonist-treated mice [51] have an increased number of MDSCs within the tumor microenvironment. It has also been seen that vitamin A-rich diets can enhance antitumor immunity rendered by the irradiated tumor cells [52], and that all-*trans*-RA can improve the antitumor protection by both tumor peptide and Ad-p53-transduced DCs in different tumor models [53]. The same effect was observed in patients with metastatic renal carcinoma [54]. Furthermore, BMDCs generated in the presence of all-*trans*-RA showed enhanced migration toward the DLN in B16 tumor model due to higher MMP but lower TIMP [30]. Although all-*trans*-RA has been widely used in "differentiation therapy" as an effective treatment for acute promyelocytic leukemia, the role of RA in regulating antitumor immunity in a solid tumor system remains to be elucidated [55]. In summary, RA may enhance protective antitumor immunity through mechanisms such as induction of cell differentiation and enhancement of migration to lymph nodes (Fig. 2). Because of the relevance in understanding the role of RA on tumor immunology, it is imperative to translate the recent discoveries in which new technologies have been used (agonists/antagonists drugs, engineered mice, etc.) to the tumor field.

3.7. RA and the response to pathogen-associated stimuli

With the emergence of RA as a central regulator of immune function, how endogenous RA synthesis is controlled and which cells produce it has become an area of intense

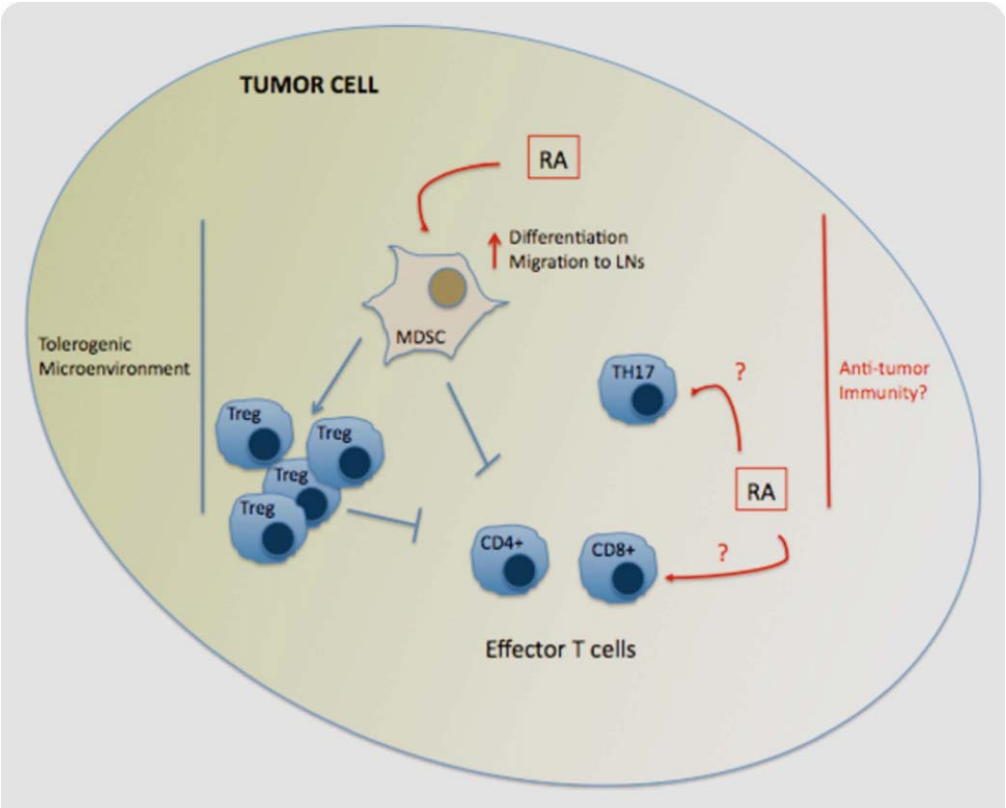

Fig. 2. Tumor cells caused by chronic inflammation lead to systemic accumulation of MDSCs and T^{regs}. Both T^{regs} and MDSCs contribute to the immunosuppressive tumor microenvironment by inhibiting both $CD4^+$ and $CD8^+$ T-cell response against the tumor. Although Th17 cells have not been confirmed in all tumor models, adoptive transfer of Th17 cell can eradicate large established tumors. RA treatment can induce MDSCs differentiation to nonsuppressive DCs. These mature DC cannot inhibit the $CD4^+$ and $CD8^+$ T-cell response against the tumor. However, the direct effect of RA on T cells in the antitumor immunity has not elucidated yet. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

interest. As mentioned earlier, gut-resident DCs have been characterized to express RALDH enzymes and produce RA. Recently, it was described that not only gut-resident DCs are able to express RALDH but also BMDCs and splenic DCs activated with inflammatory stimuli. In this regard, proinflammatory cytokines such as GM-CSF, IL-4, and toll-like receptor (TLR) agonists upregulate RALDH expression on DCs [56]. In another report, TLR5-expressing DCs responded to flagellin (a pathogen-derived component) by upregulating the expression of RALDH, promoting the development of proinflammatory Th1 and Th17 T lymphocytes and IgA^+ producing B lymphocytes [18]. This suggests that a wide spectrum of inflammatory mediators (cytokines and TLR agonists) can induce RA synthesis and this RA acts as a vital modulator of the immune system.

A related effect of RA on immunity may be associated with some of the specific genes that are up regulated by RA in hematopoietic cells. RA-inducible gene-I (RIG-I) is a member of the RIG-I-like receptors (RLRs) family, which is located in the cytoplasm and recognizes viral RNA [57]. RIG-I has been involved in the innate immune response against viruses. Trottier et al. investigated the impact of RA in the infectivity of Measles virus (MeV) on the promonocytic cell line U937. In their studies, RA-treated cells showed reduced viral infection due to the upregulation of interferon-stimulated genes. Surprisingly, bystander cells exposed to the media containing the RA-treated cells were refractory to MeV infection, suggesting that soluble factors are the effectors and that the response can be transmittable. Vitamin A or RA is known as an effective treatment against acute measles, which affects ~30 millions of children a year [58]. This report suggested a mechanism by which RA exerts antiviral activity. Recently, a new mechanism of RIG-I activation has been described. Wang et al. reported that, besides viral RNA, RIG-I can also be stimulated by the TLR4 ligand LPS. In this study, LPS-stimulated macrophages upregulate the expression of RIG-I, which allowed the expression of the proinflammatory cytokine TNF-α [59]. Interestingly, the enhanced response developed by Influenza A-infected individuals is due to the recognition of the virus through RIG-I, which upregulates type-I interferons (IFN-α and IFN-β) together with the activation of NF-κB that triggers the expression of other proinflammatory modulators as IL-6 and IL-8 [60]. In Fig. 3, we summarize the current knowledge of RA on immune cells, specifically on APCs (such as DCs) and T lymphocytes.

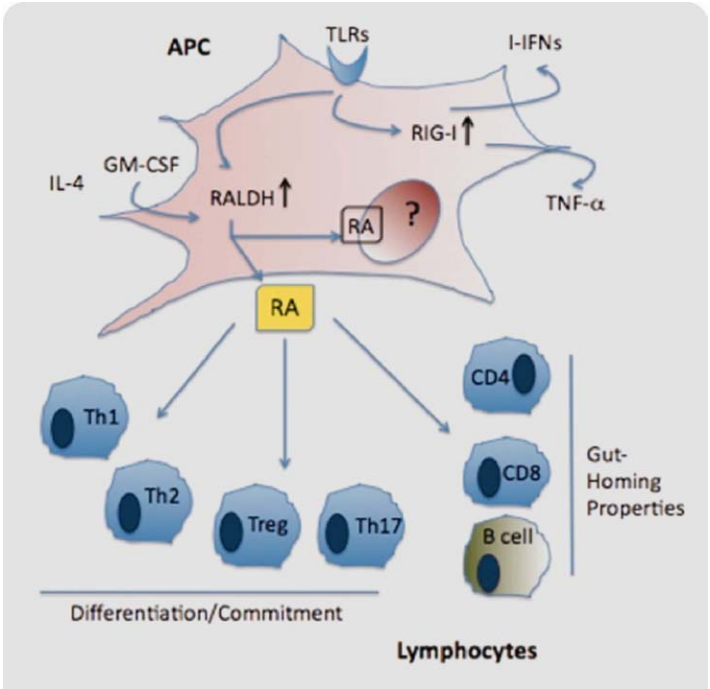

Fig. 3. RA modulates the immune response. An inflammatory microenvironment can impact and change the phenotype of APCs as a consequence RA production. RA is known for inducing gut-homing properties on T and B lymphocytes, and for influencing the generation and differentiation of different subpopulation of T lymphocytes, which are a crucial arm of the immune response. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

From the above, we can conclude that RA can exert both pro- and anti-inflammatory effects. No doubt, RA exerts these effects in ways to coordinate a balance of positive and negative effects to optimize host resistance.

4. Concluding remarks

The role of vitamins, specifically vitamin A, has been proven to be important to maintain a healthy state. In this review, we summarized and discussed the last discoveries regarding the involvement of vitamin A and RA in the immune system, underscoring its growing importance in regulating T-cell–mediated immunity. The fact that RA controls critical checkpoints in inflammation and tolerance makes it an attractive target to modulate immunity. However, additional studies are required to precisely understand how RA controls these events at a molecular level, the receptors involved and the way RA synthesis is regulated *in vivo*.

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