CHAPTER ELEVEN

Role of Retinoic Acid-Metabolizing Cytochrome P450s, CYP26, in Inflammation and Cancer

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Abstract

Vitamin A (retinol) and its active metabolite, all-trans-retinoic acid (atRA), play critical roles in regulating the differentiation, growth, and migration of immune cells. Similarly, as critical signaling molecules in the regulation of the cell cycle, retinoids are important in cancers. Concentrations of atRA are tightly regulated in tissues, predominantly by the availability of retinol, synthesis of atRA by ALDH1A enzymes and metabolism and clearance of atRA by CYP26 enzymes. The ALDH1A and CYP26 enzymes are expressed in several cell types in the immune system and in cancer cells. In the immune system, the ALDH1A and CYP26 enzymes appear to modulate RA concentrations. Consequently, alterations in the activity of ALDH1A and CYP26 enzymes are expected to change disease outcomes in inflammation. There is increasing evidence from various disease models of intestinal and skin inflammation that treatment with atRA has a positive effect on disease markers. However, whether aberrant atRA concentrations or atRA synthesis...
and metabolism play a role in inflammatory disease development and progression is not well understood. In cancers, especially in acute promyelocytic leukemia and neuroblastoma, increasing intracellular concentrations of atRA appears to provide clinical benefit. Inhibition of the CYP26 enzymes to increase atRA concentrations and combat therapy resistance has been pursued as a drug target in these cancers. This chapter covers the current knowledge of how atRA and retinol regulate the immune system and inflammation, how retinol and atRA metabolism is altered in inflammation and cancer, and what roles atRA-metabolizing enzymes have in immune responses and cancers.

**ABBREVIATIONS**

9-\textit{cis}RA 9-\textit{cis}-retinoic acid  
13-\textit{cis}RA 13-\textit{cis}-retinoic acid  
ALDH aldehyde dehydrogenase  
APL acute promyelocytic leukemia  
atRA all-trans-retinoic acid  
BCDO2 β,β-carotene-9,10-dioxygenase 2  
BCMO1 β,β-carotene-15,15'-monooxygenase 1  
CD Crohn’s disease  
CRABP cellular retinoic acid-binding protein  
CRBP cellular retinol binding protein  
CYP26 cytochrome P450 family 26  
DC dendritic cell  
ER endoplasmic reticulum  
FABP fatty acid binding protein  
GALT gut-associated lymphoid tissue  
IBD inflammatory bowel disease  
LRAT lecithin retinol acyltransferase  
NAFLD non-alcoholic fatty liver disease  
NASH non-alcoholic steatohepatitis  
PML promyelocytic leukemia gene  
PPAR peroxisome proliferator-activated receptor  
RA retinoic acid  
RAMBA retinoic acid metabolism blocking agent  
RAR retinoic acid receptor  
RBP retinol binding protein  
RDH1 retinol dehydrogenase 1  
REH retinyl ester hydrolase  
RXR retinoid X receptor  
UC ulcerative colitis

**1. INTRODUCTION**

Vitamin A is one of the fat-soluble vitamins critical for health in humans and other mammals. Typically, the term vitamin A is used to
describe retinol and retinyl esters (Fig. 1), including retinyl palmitate, that are the main dietary forms of vitamin A obtained from animal products (Schoeff, 1983; Sklan, 1987). However, retinol and retinyl esters are not biologically active but require oxidation by several enzymes to generate retinoic acid (RA), the main compound responsible for the biological activity of vitamin A. Retinyl esters serve as the reservoir of vitamin A in an organism and are stored in the body primarily in the liver stellate cells and in the lungs and adipose (Chytil, 1992; Sauvant, Cansell, & Atgïé, 2011). The esterification of retinol and the hydrolysis and release of the stored esters as retinol are regulated by the activity of two enzymes, lecithin retinol acyltransferase (LRAT) and retinyl ester hydrolase (REH), respectively (Fig. 2). The circulating retinol is taken up into target cells by an uptake transporter STRA6, but due to its high lipophilicity retinol may also cross membranes by passive diffusion. In target cells, retinol is oxidized to retinaldehyde by several alcohol dehydrogenase enzymes including retinol dehydrogenase (RDH) 1, RDH10, RDH-E, RDHE2, and DHRS9 (D’Ambrosio, Clugston, & Blaner, 2011; Napoli, 2012; Ross, 2003; Fig. 2). Alternatively, retinaldehyde can be obtained via direct cleavage of β-carotene by carotenoid cleavage enzymes (Lobo, Amengual, Palczewski, Babino, & von Lintig, 2012). Two enzymes have been indicated as the primary enzymes generating retinaldehyde, mammalian β,β-carotene-15,15’-monooxygenase 1 (BCMO1), and β,β-carotene-9,10-dioxygenase 2 (BCDO2) (Lobo et al., 2012).
To generate vitamin A from dietary carotenoids, retinaldehyde has to be reduced to retinol, and retinol is subsequently esterified and stored as esters (Fig. 2). The reduction of retinaldehyde to retinol in humans is catalyzed by several enzymes including RDH11, RDH12, RDH13, RDH14, and DHRS3 (Kedishvili, 2013). The relative contribution of these reductive enzymes to retinol synthesis from retinaldehyde is not well characterized, and the importance may vary between species, tissues, developmental stages, and age after birth. While the conversion of retinol to retinaldehyde is reversible, oxidation of retinaldehyde to RA appears to be irreversible. Four aldehyde dehydrogenase (ALDH) enzymes and aldehyde and xanthine oxidases have been reported to catalyze the oxidation of retinaldehyde to RA (Fig. 2; Ambroziak, Izaguirre, & Pietruszko, 1999; Kumar, Sandell, Trainor, Koentgen, & Duester, 2012; Taibi & Nicotra, 2007; Terao et al., 2009). In addition, several cytochrome P450 enzymes in humans
and rats oxidize retinaldehyde to RA (Raner, Vaz, & Coon, 1996; Tomita, Okuyama, Ohnishi, & Ichikawa, 1996; Zhang, Dunbar, & Kaminsky, 2000). It is generally believed that ALDH1A2 and ALDH1A3 are the predominant enzymes that form RA during fetal and embryonic development, as neither the Aldh1a2−/− nor the Aldh1a3−/− mice are viable. However, the importance of ALDH1A1 in RA synthesis is unclear as Aldh1a1−/− mice are viable and fertile (Kumar et al., 2012). Very little is known of the potential importance of ALDH8A1 in RA synthesis. Recent studies have shown that the relative importance of ALDH1A enzymes in RA synthesis is tissue specific. For example, ALDH1A1 appears to be responsible for RA synthesis in the liver while ALDH1A2 and ALDH1A1 make the main contributions to RA synthesis in the testes (Arnold, Kent, Hogarth, Griswold, et al., 2015; Arnold, Kent, Hogarth, Schlatt, et al., 2015). This tissue-specific expression is important, as locally generated RA gradients are believed to be a key factor in regulating biological processes. Yet, the overall quantitative importance of the different enzymes in retinaldehyde oxidation in different species and organs is not well characterized.

RA has five chemical isomers (all-trans-RA, 9-cis-retinoic acid (9-cisRA), 13-cis-retinoic acid (13-cisRA), 11-cisRA, and 9,13-dicisRA) (Fig. 1). Of these isomers, all-trans-RA (atRA) is considered to be the biologically active isomer and the primary enzymatic product of retinaldehyde oxidation. However, in various species including humans, the 9-cisRA, 13-cisRA, and 9,13-dicisRA isomers have also been detected in different organs (Arnold, Amory, Walsh, & Isoherranen, 2012; Kane, Folias, Wang, & Napoli, 2008). The in vivo source of these isomers is not clear. Although isomerization between all-trans and cis retinols has been characterized (McBee et al., 2000), the stereochemistry of the retinaldehyde precursor in vivo has not been determined. 9-cisRA binds to retinoid X receptors (RXRs) and this isomer may be important in some tissues such as the pancreas where it has been detected (Kane et al., 2010). Yet, 9-cisRA concentrations in vivo are typically very low or undetectable, and the biological role of this isomer has not been confirmed. In contrast, 13-cisRA is detected in all tissues and the concentrations of 13-cisRA are often higher than atRA. Yet, 13-cisRA is considered to be devoid of biological activity due to its low affinity to nuclear retinoic acid receptors (RARs) in comparison to atRA (Aström, Pettersson, Krust, Chambon, & Voorhees, 1990). In vivo, 13-cisRA isomerizes to atRA both via glutathione-S-transferase-mediated and thermodynamic processes (Chen & Juchau, 1997; Sass, Forster,
Bock, & Nau, 1994). Hence, the activity of 13-\textit{cis}RA is believed to be a result of isomerization to \textit{at}RA. In many species, including human, 9,13-\textit{dicis}RA is also found at concentrations as high as \textit{at}RA and 13-\textit{cis}RA. However, this isomer appears to be devoid of biological activity (Chen et al., 2000; Nonnecke, Horst, Dubeski, & Reinhardt, 1997; Zile, Emerick, & DeLuca, 1967). Throughout this text, the terms \textit{at}RA and 13-\textit{cis}RA are used when a specific isomer has been identified in the research, and the generic term RA is employed when the identity of the isomer has not been specified.

### 1.1 RA Signaling and Biological Activity

Retinoid signaling and \textit{at}RA have been shown to be important in various biological processes and in treatment of several diseases. For example, endogenous \textit{at}RA is critical in the maintenance of healthy skin, epithelia, and the immune system (Napoli, 2012), in regulation of bone growth and homeostasis, and in regulating continuous, asynchronous spermatogenesis (Chung & Wolgemuth, 2004; Hogarth & Griswold, 2013). In addition, \textit{at}RA is a classic morphogen that regulates embryonic development (Duester, 2008), organogenesis, stem cell differentiation (Gudas & Wagner, 2011), and body patterning in all chordates. Retinoid signaling also plays a central role in neuronal differentiation (Maden, 2007) and in tissue repair and regeneration (Gudas, 2012). In addition, retinoid signaling has been linked to regulation of insulin-stimulated glucose secretion (Chung & Wolgemuth, 2004; Kane et al., 2010, 2008) and to lipid homeostasis and adiposity (Bonet, Ribot, & Palou, 2012). Recently, \textit{at}RA has been found to also play a role in modulating the inflammation that accompanies many human diseases. Exogenous \textit{at}RA and 13-\textit{cis}RA have been shown to be beneficial in the treatment of chronic hand eczema (Ruzicka et al., 2004) and in a variety of cancers including acute promyelocytic leukemia (APL) (Tang & Gudas, 2011), neuroblastoma in children (Veal et al., 2007), and Kaposi’s sarcoma (Altucci, Leibowitz, Ogilvie, de Lera, & Gronemeyer, 2007). Whether exogenous RA functions via similar pathways and receptors as endogenous \textit{at}RA is not well defined.

The biological activity of \textit{at}RA is predominantly mediated by binding to RAR\textit{α}, RAR\textit{β}, and RAR\textit{γ}, but \textit{at}RA has also been shown to bind to peroxisome proliferator-activated receptors (PPAR\textit{β}/\textit{δ}) (Schug, Berry, Shaw, Travis, & Noy, 2007). Binding of retinoids to either RARs or PPARs results in increased transcription of respective target genes, and these regulatory
mechanisms have been thoroughly reviewed elsewhere (Gudas, 2012; Noy, 2010). The observed effects of atRA on gene transcription are dependent on the cellular concentrations of atRA as well as the expression levels of RAR and PPAR isoforms. In addition, the expression of cellular binding proteins that bind atRA, most notably cellular retinoic acid-binding proteins (CRABPs) and fatty acid binding proteins (FABPs), has been shown to direct the activity of atRA isomers toward the different nuclear receptors (Noy, 2000; Schug et al., 2007). Overall, atRA signaling in various tissues is tightly controlled by the interplay between expression levels of the nuclear receptors and binding proteins, and the expression and activity of the atRA synthesizing and eliminating enzymes that control atRA concentrations in target tissues.

1.2 Biochemistry, Expression, and Function of CYP26 Enzymes

The clearance of atRA is mediated predominantly by the cytochrome P450 family 26 enzymes (CYP26) in all chordates, although many other CYP enzymes including human CYP3A4 and CYP2C8 and rat Cyp2c22 also oxidize atRA and its 9-cisRA and 13-cisRA isomers (Marill, Cresteil, Lanotte, & Chabot, 2000; McSorley & Daly, 2000; Nadin & Murray, 1999). It is widely believed that oxidation of atRA in the four-position of the β-ionone ring to generate 4-OH-RA (Fig. 1) is the predominant route of elimination of atRA but no unequivocal mass balance studies confirm this. Determining the major route of atRA elimination in humans or animal species is challenging as 4-OH-RA is extensively metabolized both in vitro and in vivo. 4-OH-RA is glucuronidated predominantly by UGT2B7 and oxidized by CYP26s and microsomal ADHs to 4-oxo-RA and other products (Samokyszyn et al., 2000; Topletz et al., 2015). The CYP26s and other CYP enzymes also generate several other hydroxylation products from atRA (Thatcher et al., 2011). The best characterized of these additional hydroxylation products is the 18-OH-RA (Fig. 1), which has been synthesized (Rosenberger & Neukom, 1982) and shown to be a metabolite formed by CYP26A1, CYP26B1, and other CYPs (Topletz et al., 2012). In addition, 16-OH-RA (Fig. 1) has been proposed as a metabolite of atRA, but this metabolite has never been synthesized, and therefore the identification of the metabolite is based on interpretation of mass spectrometry fragmentation patterns (Thatcher et al., 2011).

The CYP26 family consists of three highly conserved enzymes CYP26A1, CYP26B1, and CYP26C1. Despite the fact that RA appears
to be the primary substrate of all three CYP26 enzymes and all CYP26s metabolize atRA predominantly to 4-OH-RA, these three enzymes share only 40–50% sequence similarity in a given species (Thatcher & Isoherranen, 2009). Yet, the individual isoforms are highly conserved across chordates which all have three CYP26 enzymes. Overall, from genomic and phylogenetic analysis it appears that CYP26 enzymes evolved very early together with RA signaling and play critical roles in regulating developmental processes. As such, it is not surprising that both Cyp26a1−/− and Cyp26b1−/− mice suffer from multiple developmental defects. The Cyp26a1−/− mice die during gestation, while Cyp26b1−/− mice survive till birth but die shortly after and have severe, but specific, malformations (Abu-Abed et al., 2001; MacLean et al., 2001; Uehara et al., 2007; Yashiro et al., 2004). Notably, the pattern of developmental defects in the two mouse models is different, pointing to the unique roles the two CYP26 enzymes play during organogenesis and body axis formation. In contrast, Cyp26c1−/− mice are viable and free of malformations although the simultaneous knockout of Cyp26c1 with Cyp26a1 aggravates the phenotype of the Cyp26a1−/− mice (Uehara et al., 2007).

Functionally, CYP26 enzymes are membrane-anchored P450 proteins that require NADPH and P450 reductase for their function. While it is theoretically possible that the CYP26 enzymes reside in the mitochondria instead of the endoplasmic reticulum (ER), the fact that the CYP26 enzymes require P450 reductase from the ER membrane to function suggests that these enzymes are ER membrane bound (Lutz et al., 2009; Topletz et al., 2012). However, the localization of the CYP26 enzymes within a cell has not been unequivocally determined. Interestingly, unlike other P450 enzymes such as CYP17, cytochrome b5 does not affect the catalytic activity of the CYP26 enzymes, and P450 reductase seems to solely support the catalytic activity of these enzymes (Lutz et al., 2009). Overall, the affinity of atRA to each of the CYP26 enzymes is high and the $K_m$ values for CYP26A1 and CYP26B1 are <100 nM for atRA. Interestingly, the $K_m$ value for atRA with the CYP26s approximates the concentrations of atRA in various tissues (Topletz et al., 2012). While the affinity of the substrate is high, the $k_{cat}$ values for atRA with CYP26 enzymes are similar to other mammalian P450 enzymes (approximately 1–10 pmol/min/pmol) (Thatcher, Zelter, & Isoherranen, 2010; Topletz et al., 2012). Since the affinity of atRA to CYP26 is approximately 1000-fold higher than that to other CYP enzymes, the overall intrinsic clearance of atRA by CYP26 enzymes is 1000- to 10,000-fold higher than other CYP enzymes such as
CYP3A4 and CYP2C8 (Thatcher et al., 2010). Therefore, even in tissues such as the human liver which have high expression levels of CYP3A4 and CYP2C8, the CYP26s are expected to be the main contributors to RA clearance even if they are expressed at low levels (Thatcher et al., 2011, 2010; Thatcher & Isoherranen, 2009). Indeed, in a study of individual donors in a human liver bank, CYP26A1 was shown to be the main enzyme contributing to arRA clearance, but the expression of CYP26A1 was subject to considerable interindividual variability (Thatcher et al., 2010). Interestingly, in the human liver CYP26B1 protein was completely absent pointing to tissue- and cell-type-specific roles of the CYP26 enzymes in adult tissues and during fetal development.

It is generally believed that RA concentrations in target tissues are regulated locally by RA synthesis and clearance, and that systemic RA concentrations do not reflect RA homeostasis in specific tissues. Therefore, extrahepatic expression of CYP26 enzymes is expected and likely plays a role in tissue-specific clearance and regulation of RA concentrations. Indeed, CYP26A1 and CYP26B1 mRNA and protein are found in multiple extrahepatic human tissues (Topletz et al., 2012; Xi & Yang, 2008) with CYP26B1 appearing to have a higher expression in extrahepatic tissues than CYP26A1. In agreement with the human tissue data of CYP26 expression, metabolism of arRA was shown in rat testes, kidney, and lung microsomes (Fiorella & Napoli, 1994). In addition, CYP26 enzymes have been suggested to constitute a tissue barrier for RA distribution from circulation to specific organs. This hypothesis originated from the observation that uptake of administered radiolabeled RA to some tissues, such as the testes, pancreas, and spleen, was limited (Ahluwalia, Gambhir, & Sekhon, 1975; Kurlandsky, Gamble, Ramakrishnan, & Blaner, 1995; McCormick, Kroll, & Napoli, 1983; Smith, Milch, Muto, & Goodman, 1973), and that CYP26 enzymes were detected in several barrier cells (Heise et al., 2006; Vernet et al., 2006; Xia, Ma, Sun, Yang, & Peng, 2010). The metabolic barrier provided by CYP26 enzymes in some tissues such as the testes likely ensures that RA gradients are regulated by enzyme expression and activity within the tissue and not by circulating concentrations. Therefore, understanding the activity and expression of CYP26 enzymes in individual tissues and cell types is critically important for defining the relationship between arRA concentrations and biological outcomes within a tissue. Based on tissue-specific expression and activity of CYP26 enzymes, it can be hypothesized that tissue-specific alterations in CYP26 activity will alter arRA concentrations in the given tissue and result in altered arRA signaling. For example, inhibition of CYP26s
or genetic polymorphisms that decrease CYP26 activity would be expected to increase \( a\text{-}\text{RA} \) concentrations within the tissue in which CYP26s are expressed. On the other hand, induction of CYP26s or increased activity of CYP26 enzymes within an organ is expected to decrease the overall exposure to \( a\text{-}\text{RA} \) and possibly increase exposure to \( a\text{-}\text{RA} \) metabolites that may also have pharmacological activity. Yet, a direct effect of decreased or increased CYP26 activity and \( a\text{-}\text{RA} \) concentrations in a tissue has never been demonstrated, even though P450 inhibitors such as talarozole and liarozole that also inhibit CYP26 do increase RA concentrations in various tissues and plasma (Nelson, Buttrick, & Isoherranen, 2013; Stoppie et al., 2000; Van Wauwe, Coene, Goossens, Cools, & Monbaliu, 1990). Likewise, specific effects of CYP26A1 or CYP26B1 inhibition have never been shown to have different effects in different tissues.

In addition to metabolic enzymes, small binding proteins of the class of lipid binding proteins such as CRABP-I and CRABP-II that bind \( a\text{-}\text{RA} \) with high affinity (\( K_d = 0.06 \text{ nM} \) for CRABP-I and \( K_d = 0.13 \text{ nM} \) for CRABP-II) (Dong, Ruuska, Levinthal, & Noy, 1999) are believed to alter \( a\text{-}\text{RA} \) homeostasis and CYP26 activity. However, CRABP\(^{-/-}\) mice are viable and devoid of any malformations or negative health effects (De Bruijn et al., 1994; Lampron et al., 1995) putting the biological significance of the binding proteins to question. CRABP-II has been shown to deliver \( a\text{-}\text{RA} \) to RAR in the nucleus whereas CRABP-I may serve a different function such as delivering \( a\text{-}\text{RA} \) to the CYP26 enzymes. Whether these functions affect overall \( a\text{-}\text{RA} \) homeostasis is still unknown.

2. ROLE OF RA IN THE IMMUNE SYSTEM

For nearly 100 years, vitamin A has been recognized as a critical nutritional component needed to support immunity. It is well established that vitamin A deficiency results in increased morbidity and mortality from certain pathogens, and several clinical trials have shown that vitamin A supplementation in at risk populations improves overall outcomes (Ross, 2012). In support of human studies, vitamin A-deficient mice were shown to be completely deficient in CD4\(^+\) and CD8\(^+\) T-cells in the submucosal lamina propria in the intestine (Iwata et al., 2004). This suggests that gut mucosal immunity is impaired in vitamin A deficiency, and potentially explains the specific link between poor outcomes in diarrheal disease and vitamin A deficiency. Yet, it is possible that retinol and retinol binding protein (RBP) also contribute to regulation of gut mucosal immunity either
together with RA or independent of RA as retinol and RBP concentrations are significantly decreased in vitamin A deficiency (De Pee & Dary, 2002; Gamble et al., 2001) and retinol and RBP have been shown to have activity in the absence of RA via binding to STRA6 (Berry, Levi, & Noy, 2014; Marwarha, Berry, Croniger, & Noy, 2014). However, current evidence suggests that atRA itself is critical in maintaining immune homeostasis (Hall, Grainger, Spencer, & Belkaid, 2011; Raverdeau & Mills, 2014), is the required signaling molecule in the regulation of T-cell, B-cell, and dendritic cell (DC) differentiation and function, and in the production of both pro- and anti-inflammatory cytokines (Hansen, Westendorf, & Buer, 2008; Raverdeau & Mills, 2014).

Vitamin A deficiency, which presumably results in lower concentrations of atRA, promotes naïve CD4+ T-cell differentiation into Th1 cells, which secrete interferon-γ (Cantorna, Nashold, & Hayes, 1995). High concentrations of atRA, on the other hand, generally promote T-cell differentiation into Th2 cells producing IL-4 and IL-5 (Fig. 3). Hence, it appears that

![Figure 3](image)

**Figure 3** Retinoic acid in steady-state immune homoestasis. (A) Throughout the body, RA plays an important role in the ratio of Th1:Th2 cells by reducing differentiation of naïve T-cells into IFN-γ producing Th1 cells and promoting differentiation of Th2 cells, which secrete IL-4 and IL-5. (B) In gut-associated lymphoid tissue, dendritic cells (DCs) synthesize RA, which acts synergistically with TGF-β to initiate differentiation of Foxp3+ Treg cells and suppress T-cell differentiation into Th17 cells. RA produced by DCs also induces the expression of α4β7 and CCR9 on naïve T-cells to prompt gut homing to the lamina propria over peripheral lymph nodes. Cytokines shown are not a complete list but were chosen based on relevance to the text.
increasing concentrations of atRA in the precursor T-cell environment will increase the Th2 to Th1 cell ratio (Ross, 2012). In addition, of interest especially to gut mucosal immunity, atRA also regulates Th17 cell function by opposing Th17 cell commitment and inhibiting T-cell differentiation into Th17 cells (Mucida et al., 2007; Fig. 3). RA synthesis by the DCs in the gut-associated lymphoid tissue (GALT) is believed to initiate Treg cell differentiation. RA produced by DCs in GALT also induces the expression of α4β7 and CCR9 on T-cells upon antigen presentation (Iwata et al., 2004) inducing gut homing of the T-cells to the lamina propria over peripheral lymph nodes (Fig. 3). Activation of naïve T-cells in the presence of RA induced CCR9 and the α4 integrin as well (Kang, Park, Cho, Ulrich, & Kim, 2011). In Treg cell activation, RA acts synergistically with TGF-β to enhance the expression of Foxp3, a critical transcription factor for Treg cells (Hall et al., 2011; Mucida et al., 2007). The effects of atRA on differentiation and homing of Treg cells to the gut mucosa and inhibition of Th17 cell formation appear to be regulated via RARα (Mucida et al., 2007; Raverdeau et al., 2014). Hence, increasing RA concentrations is expected to prevent the induction of autoimmune T-cells and modulate inflammatory responses. Yet, many of the responses observed with RA appear to be dependent on the cytokine environment. Overall, RA plays a critical role in modulating immune responses and inflammation, and regulation of RA concentrations is likely important in inflammatory and autoimmune diseases. However, how RA concentrations are controlled within the immune system is not well established, and it is possible that the roles of the individual enzymes are divergent in different inflammatory conditions and in different tissues.

Based on current data, the DCs in the GALT express RDH and ALDH1A enzymes and produce RA (Iwata et al., 2004). The elimination of atRA also appears to be regulated within the immune system as CYP26 enzymes are expressed and inducible by atRA in T- and B-cells (Takeuchi, Yokota, Ohoka, & Iwata, 2011; Wen et al., 2013). Interestingly, in naïve T-cells in vitro, TGF-β inhibits and TNF-α enhances CYP26B1 induction by atRA (Takeuchi et al., 2011). CYP26B1 mRNA was found in T-cells from mouse GALT, including mesenteric lymph nodes and Payer’s patches, but not from peripheral lymph nodes (Takeuchi et al., 2011). CYP26A1 and CYP26B1 mRNA expression and RA-dependent induction have also been demonstrated in B-cells of healthy humans (Wen et al., 2013). In vitro, when T-cells from mice were stimulated under conditions promoting T-cell differentiation, CYP26B1 expression was significantly increased in differentiated Th17 cells but was significantly lower in
differentiated Treg cells and lowest in undifferentiated T-cells suggesting that depletion of RA by CYP26B1 regulates RA concentrations within the T-cells (Chenery et al., 2013). A higher expression of CYP26B1 should result in lower RA concentrations within the cells removing the RA signaling. However, somewhat surprisingly when CYP26B1 was knocked out in the T-cells, the frequency of both IL-17 producing T-cells (Th17 cells) and FoxP3+ Treg cells was increased (Chenery et al., 2013). Based on these data, it is likely that alterations in the activity of ALDH1A and CYP26 enzymes within the immune system are a key factor modulating aTRA concentrations and signaling that subsequently drives immune and inflammatory responses.

### 3. CYP26 AND RA IN CHRONIC INFLAMMATORY DISEASES

A large portion of human diseases are associated with chronic inflammation. In some of these conditions inflammation is related to the primary pathology of the disease, whereas in others only clinical associations between disease progression and inflammatory markers have been observed. For example, non-alcoholic fatty liver disease (NAFLD) is defined as an excess accumulation of lipids in the liver but progression to non-alcoholic steatohepatitis (NASH) is thought to be a result of triglyceride and cholesterol accumulation in the liver leading to oxidative stress, insulin resistance, and inflammation (Depner, Philbrick, & Jump, 2013). As RA and RAR activation is known to play an important role in the liver and RA is an important regulator of inflammation, it is possible that development of NASH and associated inflammation involves altered aTRA signaling. In the case of NAFLD and NASH, analysis of mRNA from patient liver biopsies showed increased mRNA levels of a variety of enzymes involved in regulation of aTRA and vitamin A concentrations including CRBP1, LRAT, RDH5, RDH11, DHR53, ALDH1, ALDH3, and CYP26A1 (Ashla et al., 2010). However, whether any of these changes are associated with altered liver aTRA concentrations, play a role in disease development and progression, or are a result of liver disease is not known.

Perhaps the best-demonstrated connections between chronic inflammatory markers and disease progression have emerged from autoimmune diseases. Autoimmune diseases arise from an abnormal immune response of the body against its own tissues or substances (Abella et al., 2014; Feldmann, Brennan, & Maini, 1996). Treatment of these diseases generally focuses on regulation of immune system activity. While autoimmune diseases as well
as inflammatory conditions are a large group of diseases, this section focuses on those conditions in which atRA signaling and CYP26 activity have been shown to be altered or potentially play a role in modulating the disease progression.

### 3.1 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is an inflammatory disease of the gastrointestinal tract and a link may exist between regulation of intestinal immunity by RA and IBD progression. The principal forms of IBD are Crohn’s disease (CD) and ulcerative colitis (UC). The pathology of these diseases is complicated and thought to arise as a result of environmental, genetic, and immunologic factors. Both CD and UC are associated with an excessive response of mucosal adaptive and innate immunity (Brown & Mayer, 2007; Talley et al., 2011). In active IBD, increased levels of macrophages and pro-inflammatory cytokines are found in intestinal mucosa (Mahida, 2000). Ex vivo studies in cells from UC patients and in vivo mouse studies have found that increasing atRA concentrations could overcome the dysfunction of the immune system and ameliorate inflammation. For example, in an in vitro colitis model using murine macrophages, atRA treatment decreased the activation of NFκB, a macrophage nuclear transcription factor, and production of the pro-inflammatory cytokine TNF-α. In a mouse model of colitis, atRA treatment reduced symptoms of inflammation including reduced infiltration of neutrophils, decreased levels of NFκB, and lower levels of TNF-α in colon tissues in comparison to control mice (Hong et al., 2014). Similar to mouse models of colitis, treatment of cultured colonic mucosa cells from UC patients with RA decreased concentrations of TNF-α, upregulated Foxp3 and downregulated IL-17, returning immunologic balance. The nuclear receptor RARα was implicated in regulating these RA effects (Bai et al., 2009). These studies did not evaluate whether RA was a priori decreased in the disease setting due to either decreased synthesis or increased metabolism of RA. However, these studies suggest that increasing levels of atRA may be effective in halting the acute inflammatory response in IBD patients.

Further evidence of the potential benefit of increased RA in IBD comes from studies in T-cell-specific CYP26B1−/− mice (Chenery et al., 2013). Although RA concentrations were not measured, it is expected that CYP26B1−/− T-cells have increased RA concentrations compared to
wild-type mouse T-cells. When naïve CD4+ T-cells from T-cell-specific CYP26B1−/− mice and control mice were implanted into a murine model of T-cell-dependent intestinal inflammation, inflammation and disease progression were attenuated in the presence of CYP26B1−/− T-cells versus control (Chenery et al., 2013). Still, T-cell-specific CYP26B1−/− mice had equivalent T-cell development as controls. In addition, IL17α and TNF-α mRNA levels were decreased in the colon of mice with CYP26B1−/− T-cells but Foxp3 mRNA was unaffected by the knockout. Assuming that CYP26B1 metabolizes RA within the T-cells these findings are consistent with decreased Th17 cells and unaltered Treg cells. The findings in the T-cell-specific CYP26B1−/− mice suggest that CYP26B1 plays an important role in modulation of T-cell homeostasis. Surprisingly, the expression of RA-inducible homing signals α4β7 and CCR9 was not affected in the T-cell-specific CYP26B1−/− mice (Chenery et al., 2013).

In addition to the mouse T-cell-specific CYP26B1−/− model, the effect of CYP26B1 polymorphisms on CD and IBD risk was studied (Fransén et al., 2013). The CYP26B1 T to C polymorphism (rs2241057) that results in L264S amino acid change displays enhanced catabolic activity compared to the T allele suggesting concentrations of RA may be higher in patients with the T allele (Fransén et al., 2013). The frequency of the lower activity T allele was not significantly higher in CD patients compared UC patients or healthy controls. However, atRA concentrations were not measured in this study and no firm relationship between the polymorphism, RA levels and risk of developing CD could be made.

Despite a large volume of data supporting the concept that increasing RA levels would combat IBD, it has also been proposed that increased gut RA levels contribute to IBD pathogenesis. This suggestion is based on the observation that ALDH1A mRNA was increased in intestinal mucosa of CD patients compared to controls. Specifically, ALDH1A1 was predominantly expressed in macrophages from patients and ALDH1A2 was found in DCs (Sanders et al., 2014). While increased ALDH1A mRNA in CD patients suggests that formation of atRA in some or all subsets of antigen-presenting cells in intestinal mucosa is increased, altered RA concentrations, or increased RA synthesis was not measured in this study. Hence, it is possible that ALDH1A expression is increased in the intestinal mucosa as a compensatory mechanism to low atRA or vitamin A concentrations. Overall further studies that directly measure RA concentrations in IBD and CD are needed to determine the role of atRA signaling in these diseases.
3.2 Psoriasis

Psoriasis is characterized by an abnormally excessive growth and aberrant differentiation of the epidermal layer of skin. In psoriasis, premature maturation of keratinocytes is induced by an inflammatory cascade originating from DCs, macrophages, and T-cells (Nestle, Kaplan, & Barker, 2009). The beneficial effect of oral and topical retinoids in psoriasis is well established. In general, retinoids have an anti-proliferative effect in epidermal cells if the microenvironment is hyperproliferative and a proliferative effect if the microenvironment is normal (Harper, 1988; Harper & Burgoon, 1982). In normal cells, RA stimulates keratinocyte growth and differentiation, while in psoriasis RA is anti-proliferative. In psoriasis, retinoid treatment decreases cytotoxic T-cell induction, inhibits the chemotactic responses of activated lymphocytes, and inhibits expression of migration inhibitory factor-related protein-8 (a marker for hyperproliferation of keratinocytes) resulting in improvement in patient symptoms (DiSepio, Malhotra, Chandraratna, & Nagpal, 1997; Dupuy, Bagot, Heslan, & Dubertret, 1989; Van de Kerkhof, 2006).

Similar to RA signaling in gut mucosal immunity, RA signaling is important in the DCs and T-cells in the skin. Similar to DCs in the GALT, DCs in the skin have been shown to express ALDH1A enzymes and to produce RA (Guilliams et al., 2010). Analogously to the gut mucosa, DCs in the skin were shown to induce Treg cell differentiation and Foxp3 expression in ALDH1A expression-dependent manner (Guilliams et al., 2010). Interestingly, in contrast to the DCs in the GALT, DCs in the skin do not induce the gut homing signals such as CCR9 in CD4+ T-cells despite the fact that skin DCs also synthesize RA, demonstrating that gut homing signal generation is specific to GALT DCs (Guilliams et al., 2010; McCully et al., 2012).

As in most other cells of the body, retinoid levels in human skin are under tight control. Retinoids are stored in the skin as retinyl esters and RA is produced locally in the epidermis (Fisher & Voorhees, 1996; Pavez Loriè, Li, Vahlquist, & Törmä, 2009). Expression of CYP26A1 has been confirmed in the basal layer of normal and psoriatic human epidermal keratinocytes with immunofluorescence and immunohistochemical staining (Heise et al., 2006). In addition to CYP26A1, another RA-metabolizing enzyme, CYP2S1, has been identified in keratinocytes in psoriatic plaques and normal human skin (Smith et al., 2006) but the quantitative role of CYP2S1 in atRA clearance is not known. Still, the expression of two RA-metabolizing enzymes is indicative of the importance of RA homeostasis in skin.
Natural and synthetic retinoids have been used in the oral and topical treatment of psoriasis since the 1970s (Dogra & Yadav, 2014; Roos, Jugert, Merk, & Bickers, 1998). While clinical results have been positive, severe side effects such as teratogenicity, hypertriglyceridemia, and dermatitis can be dose limiting. Thus, the use of inhibitors of RA metabolism, that is, RA metabolism blocking agents (RAMBAs) has also been investigated as a means to increase endogenous RA levels in psoriasis and avoid complications from exogenous administration (Verfaille, Borgers, & van Steensel, 2008). Although RAMBAs were originally developed before the identification of CYP26 enzymes, it is now assumed that CYP26s are the primary target of these compounds (Nelson et al., 2013). Treatment of keratinocyte cultures with CYP26 inhibitors, such as talarozole (also known as R.115866 or rambazole), has been shown to potentiate cell differentiation effects of RA (Giltaire et al., 2009). At the same time, treatment with RAMBAs induced only CYP26 expression, whereas treatment with RA altered multiple enzymes in the retinoid synthesis pathway (Pavez Loriè, Chamcheu, Vahlquist, & Törmä, 2009; Smith et al., 2006). These results indicate that inhibition of CYP26 may achieve the same therapeutic benefits as exogenous RA administration without the side effects observed with atRA administration. However, it is not clear how CYP26 inhibitors will affect the immune system and inflammation in the skin.

Clinical studies with CYP26 inhibitors have also been reported in psoriasis. Patients treated with oral ketoconazole, a broad CYP inhibitor that also inhibits CYP26s, had some improvement in their scalp psoriasis. However, the trial was discontinued due to concerns of drug toxicity (Farr, Krause, Marks, & Shuster, 1985). A second-generation RAMBA, liarozole, was developed with more specific activity towards CYP26, and it was effective in treating psoriasis in early clinical trials (Bhushan et al., 2001; Dockx, Decree, & Degreer, 1995). Further improvement in CYP26 selectivity and potency led to the development of talarozole (Pavez Loriè, Li, et al., 2009). Oral administration of talarozole significantly improved psoriasis symptoms (noted as a reduction in the Psoriasis Area Severity Index or plaque severity) in Phase I and II clinical trials. Increases in plasma concentrations of atRA were observed in a single-dose escalation trial of talarozole, but RA concentrations were within or slightly above normal physiological concentrations. Talarozole was well tolerated with no severe side effects noted in Phase I or II studies (Bovenschen et al., 2007; Verfaille et al., 2007). Additionally, topical treatment with talarozole was shown to increase CRABP2 and keratin 4 and decrease keratin 2 (markers of increased RA activity) in epidermis in
healthy volunteers (Pavez Lorie`, Cools, et al., 2009). Collectively, these data demonstrate the important role of CYP26 in regulation of RA metabolism in the skin and in psoriasis. Unfortunately, none of the studies with RAM-BAs have assessed RA concentrations in the skin or inflammatory markers. Hence, it is not possible to determine whether modulation of inflammatory responses also contributes to clinical improvement.

3.3 Atherosclerosis

Atherosclerosis is a slowly progressing chronic inflammatory disease of blood vessels characterized by accumulation of lipids in the arterial wall (Hansson & Hermansson, 2011; Hansson, Robertson, & Soderberg-Naucler, 2006). Atherosclerosis is a complex disease resulting from multiple risk factors such as excess lipid accumulation, coagulation and fibrosis, cell proliferation, and inflammatory cell migration to the inflamed sites. During early stages of atherosclerosis, vascular smooth muscle cells migrate to the intima, promote extracellular matrix deposition, and reduce the lumen size (Rhee, Nallamshetty, & Plutzky, 2012). However, inflammation, including innate and adaptive immune responses, is involved in all stages of the development of atherosclerosis (Libby, Ridker, & Hansson, 2009; Wiedermann et al., 1996).

RA has diverse biological effects on arterial wall function and plays an important role in the coagulation system. Treatment of human aortic smooth muscle cells with atRA decreased cell proliferation, cell migration to the lumen, and extracellular matrix formation in the lumen (Johst et al., 2003). Additionally, administration of atRA attenuated pro-coagulant properties, promoted fibrinolysis, reduced the induction of monocytes and tissue factor-dependent arterial thrombus formation and suppressed the expression of vasoconstrictor thromboxane A2 in vascular smooth muscle cells (Barstad, Hamers, Stephens, & Sakariassen, 1995; Bulens et al., 1995; Van Giezen, Boon, Jansen, & Bouma, 1993; Watanabe et al., 2002).

Several enzymes involved in retinoid metabolism and action are expressed in the vasculature including alcohol dehydrogenases and RARs (Allali-Hassani et al., 1997; Miano, Topouzis, Majesky, & Olson, 1996), and RAR activity in human smooth muscle cells was confirmed in transient transfected reporter gene assays (Ocaya et al., 2011). Of the three CYP26 enzymes, CYP26B1 was found to be constitutively expressed in human vascular smooth cells and play a major role in atRA clearance in the arterial wall (Elmabsout et al., 2012; Krivospitskaya et al., 2012; Ocaya et al., 2011).
Based on this, RA may also play a role in regulating other functions in the vasculature. In human atherosclerotic arteries CYP26B1 mRNA expression was increased suggesting that RA concentrations are decreased in atherosclerosis. Murine atherosclerotic model arteries also expressed higher CYP26B1 mRNA than normal arteries (Krivospitskaya et al., 2012). Additionally, based on immunohistochemistry of arterial biopsies from atherosclerotic patients CYP26B1 colocalizes with CD68, a marker for activated macrophages, in atherosclerotic lesions (Krivospitskaya et al., 2012). The accumulation of CYP26B1-expressing macrophages may be the reason for increased CYP26B1 mRNA in the atherosclerotic arteries. When the frequency of the CYP26B1 SNP (L264S) with higher catalytic activity than wild type was tested in atherosclerotic patients and matched controls, no association of the variant allele expression between patients and matched controls and basic clinical characteristics was detected. However, patients who carried the minor allele variant and may have lower levels of atRA had a small increase in atherosclerotic lesion size (Krivospitskaya et al., 2012). Expression of CYP26B1 and RARβ was enhanced in human aortic smooth muscle cells treated with atRA alone or with talarozole and atRA (Ocaya et al., 2011). Talarozole together with atRA treatment also increased cellular atRA concentrations and decreased DNA synthesis and cell proliferation compared to cells treated with atRA alone. In addition, transfection with CYP26B1 siRNA led to increased atRA-mediated RAR/RXR signaling.

4. CYP26 AND RA IN CANCER

Vitamin A and RA play a crucial role in regulating cell proliferation, differentiation, and apoptosis, and as early as 1925 an association between vitamin A deficiency and carcinogenesis was suggested (Wolbach & Howe, 1925). Subsequent epidemiological, preclinical, and clinical studies established a relationship between vitamin A deficiency and a higher incidence of carcinogenesis in both humans and rodents (Basu, Temple, & Hodgson, 1988; Chaudhy, Jafarey, & Ibrahim, 1980; Dogra, Khanduja, & Gupta, 1985; Ito, Gajalakshmi, Sasaki, Suzuki, & Shanta, 1999; Stehr et al., 1985). In addition, abnormal RA metabolism potentially leading to altered RA levels has also been implicated in tumorigenesis (Moulas, Gerogianni, Papadopoulos, & Gourgoulianis, 2006; Osanai & Petkovich, 2005).

Altered expression of proteins controlling RA concentrations has been associated with multiple cancers but changes in RA concentrations have
not been directly measured in cancers. Significantly, higher activity of alcohol dehydrogenase enzymes has been shown in breast, ovarian, colorectal, and cervical cancer tissues compared to healthy human tissues (Jelski, Chrostek, Szmitkowski, & Markiewicz, 2006; Jelski, Zalewski, Chrostek, & Szmitkowski, 2004; Orywal, Jelski, Zdrodowski, & Szmitkowski, 2011, 2013). ALDH1A3 expression was also high in breast cancer tissues and ALDH1A3 levels significantly correlated with distant metastasis (Qiu et al., 2014). Further, ALDH1A1 expression was observed in 63.0% (92 of 146) of human breast invasive ductal carcinoma tissues (Zhong et al., 2013). It is possible that increases in alcohol dehydrogenase and ALDH enzymes will lead to increased RA concentrations. However, it is also possible that low RA concentrations in cancer prevent RA-mediated regulation of these enzymes or that alcohol dehydrogenase and ALDH1A enzymes are increased in an attempt to correct low RA concentrations in cancer.

The role of the retinol transport proteins RBP and STRA6 in cancer development and progression is still unclear. In human breast and colon cancers STRA6 and RBP mRNA levels were elevated in comparison to normal breast and colon tissue, and higher expression of STRA6 and RBP correlated with oncogenic properties and tumor progression suggesting an oncogenic role of STRA6 (Berry et al., 2014). In agreement, binding of holo-RBP4 (RBP4-ROH) to STRA6 was shown to trigger phosphorylation of STAT3, resulting in recruitment and activation of the JAK/STAT pathway and increased oncogenic properties in tumor cell lines (Berry et al., 2014). In contrast, in human keratinocytes, knockdown of STRA6 was shown to increase keratinocyte proliferation and lead to thickening of the epidermal layer and increased proliferation (Skazik et al., 2014). Similarly, increased expression of STRA6 increased apoptosis in normal and cancer cells and influenced p53-mediated apoptosis in bladder, ovarian, and colon cancer cell lines (Carrera et al., 2013). Overall, the findings are controversial and further work is needed to determine how STRA6 expression and RA signaling affect cancer development and oncogenesis. Additionally, down-regulation or loss of CRBP1, which regulates retinoid uptake, intracellular availability, and metabolism, has been shown in both human breast and ovarian cancer (Cvetković, Williams, & Hamilton, 2003; Kuppumbatti, Bleiweiss, Mandeli, Waxman, & Mira-Y-Lopez, 2000). Together, these studies show that proteins controlling RA signaling play an important role in many cancers.
Increased expression of CYP26 enzymes in cancers, such as breast, colorectal, and head and neck cancers has recently been shown in several studies (Brown et al., 2014; Osanai & Lee, 2014; Osanai, Sawada, & Lee, 2009). Elevated CYP26 levels in cancer tissue resulting in RA deficiency have also been linked to carcinogenesis in epidemiological studies (Berlin Grace, Niranjali Devaraj, Radhakrishnan Pillai, & Devaraj, 2006; Moulas et al., 2006). CYP26A1 was shown to be highly expressed in 42% (27/65) of surgically resected breast cancer specimens (Osanai & Lee, 2014). CYP26A1 expression was significantly higher in metastatic carcinomas ($n=10$) than in normal breast tissue suggesting that high expression of CYP26A1 is associated with decreased disease-free and overall survival in breast cancer (Osanai et al., 2014). Similarly, CYP26A1 and CYP26B1 expression was significantly increased in primary colorectal cancer compared with normal colonic mucosa (Brown et al., 2014), and CYP26A1 expression was significantly higher in sporadic human colon tumor samples than in normal tissues (Shelton et al., 2006). In addition to breast and colorectal cancer, CYP26A1 overexpression might also contribute to the development and progression of cervical malignancies and squamous neoplasia of the head and neck. For example, strong expression of CYP26A1 was observed in 19 of 50 (38%) cervical squamous cell carcinoma cases but no expression was found in normal cervical epithelium. In addition, strong expression of CYP26A1 was shown in 52 of 128 (41%) head and neck cancer cases with no CYP26A1 expressed in non-neoplastic tissues of the head and neck (Osanai et al., 2014). In Barrett’s carcinogenesis, a significant upregulation of CYP26A1 was observed in adenocarcinoma compared to non-dysplastic Barrett’s tissues (Chang, Hong, Lao-Sirieix, & Fitzgerald, 2008). Finally, CYP26A1 expression was significantly enhanced in primary ovarian cancers in comparison to normal ovarian tissue (Downie et al., 2005). While these studies show that CYP26 expression is elevated in various types of cancer, the underlying mechanism resulting in elevated CYP26 expression in human cancers and the exact role of CYP26 in carcinogenesis has not been fully clarified.

In vitro studies with CYP26 transfection to induce overexpression of CYP26 have been conducted and offer insights into the role of CYP26 in carcinogenesis (Chang et al., 2008; Xia et al., 2010). Increases in anti-apoptotic signals and oncogenes (e.g., epidermal growth factor receptor and c-Myc) were observed in dysplastic Barrett’s cells transfected with CYP26A1 in comparison to control cells. Cells overexpressing CYP26A1

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also exhibited increased proliferation (Chang et al., 2008). In addition, downregulation of p53, an apoptotic signal and tumor suppressor gene was found following overexpression of CYP26A1 in endometrial stromal cells (Xia et al., 2010). Based on these studies, use of RAMBAs to inhibit CYP26A1 and increase tumor RA levels is being pursued as a drug target in cancer (Nelson et al., 2013).

Efforts to synthesize RAMBAs with increased potency and favorable pharmacokinetics that selectively inhibit CYP26s are ongoing in several laboratories and details of these compounds can be found in several recent reviews (Nelson et al., 2013; Njar et al., 2006). These CYP26 inhibitors could potentially be used alone to increase RA concentrations or in combination with RA treatment to combat resistance to treatment and increase potency of RA. Preclinical and early clinical data for CYP26 inhibitors are promising (Godbole, Purushottamachar, Martin, Daskalakis, & Njar, 2012; Gomaa et al., 2011). Of the well-studied RAMBAs, liarozole, an inhibitor of CYP26 (IC$_{50}$ 2.2–6.0 μM), reduced tumor growth in androgen-dependent and androgen-independent rat prostate carcinoma models. Liarozole also decreased risk of death compared to cyproterone acetate in patients with metastatic prostate cancer in relapse after first-line endocrine therapy (Njar et al., 2006; Stearns, Wang, & Fudge, 1993). Another CYP26 inhibitor R116010, a benzothiazolamine, inhibited $\text{at}RA$ metabolism in intact T47D human breast cancer cells with an IC$_{50}$ value of 8.7 nM (Van Heusden et al., 2002).

4.1 Acute Promyelocytic Leukemia

Leukemia results from an uncontrolled increase in the number of immature white blood cells. A rare subset of leukemia, APL, is definitively marked by a chromosomal translocation affecting the RAR$\alpha$ gene (Martens et al., 2010). The predominant translocation, occurring in 90–95% of APL patients, is t(15;17) where chromosome 15 maps to the promyelocytic leukemia gene (PML) and chromosome 17 to the RAR$\alpha$ gene (Kakizuka et al., 1991; Orfali, McKenna, Cahill, Gudas, & Mongan, 2014). The t(15;17) translocation ultimately results in a PML–RAR$\alpha$ fusion protein (Guidez et al., 1998; Kakizuka et al., 1991; Pandolfi et al., 1991), which binds with RXR to the RA response element of target genes (Martens et al., 2010) ultimately blocking myeloid differentiation and leading to leukemogenesis (Collins, 2002; Guidez et al., 1998; Fig. 4). With wild-type RAR$\alpha$, physiological levels of $\text{at}RA$ are sufficient to dissociate negative corepressors but PML–RAR$\alpha$
Fusion proteins are less sensitive to all-RA and pharmacological levels of all-RA are required to dissociate negative corepressors from the fusion protein for gene transcription and terminal myeloid differentiation (Guidez et al., 1998).

Treatment of APL with all-RA began in the 1980s and remains standard of care to date (Huang et al., 1988; Watts & Tallman, 2014). all-RA treatment causes terminal myeloid differentiation, degradation of the fusion protein and autophagy of APL blasts. APL is highly curable with approximately 90% complete remission following front-line therapy with all-RA in combination with either anthracycline-based chemotherapy or arsenic trioxide (Tallman & Altman, 2009; Watts & Tallman, 2014). However, despite

Figure 4 Retinoic acid and CYP26 signaling in acute promyelocytic leukemia. In acute promyelocytic leukemia, a chromosomal translocation forms the PML–RARα fusion protein which binds DNA as either a homodimer or multimeric complex with RXR. (A) Physiological levels of the signaling molecule, RA, are insufficient to bind and promote release of corepressors from the complex which blocks gene transcription (including the gene for CYP26) and promotes leukemogenesis. (B) Pharmacological levels of all-RA bind the receptor, cause release of corepressors, and recruit activators for gene transcription ultimately resulting in terminal myeloid differentiation.
the high remission rates with front-line treatment of APL, relapse occurs in 10–30% of patients (Idres, Marill, & Chabot, 2005; Kelaidi et al., 2006). Several factors have been proposed to contribute to the relapse in APL patients, including mutations of the PML–RARα fusion protein blocking transcriptional activation, increased binding affinity of corepressors to the fusion protein, and increased atRA metabolism due to induction of CYP26 (Guidez et al., 1998; Idres et al., 2005). Autoinduction of atRA metabolism and resulting resistance to atRA is the best-characterized mechanism of APL relapse. In clinical pharmacokinetic studies, in APL patients exposure to atRA was significantly decreased at relapse when compared to exposure after the first dose (Muindi et al., 1992). Doubling the clinical dose of atRA at relapse was insufficient to match initial atRA exposures. Blasts cells were collected from patients at relapse and were sensitive in vitro to atRA differentiation and maintained the PML–RARα fusion protein, indicating that decreased systemic exposure to atRA was the root cause of relapse. The authors proposed that autoinduction of a CYP enzyme responsible for atRA metabolism occurred with prolonged treatment with atRA (Muindi et al., 1992). This proposal was further confirmed by the observation that pretreatment with fluconazole, an inhibitor of CYP enzymes, caused over fourfold increase in atRA AUC in APL patients (Schwartz, Hallam, Gallagher, & Wiernik, 1995). After identification of CYP26 as the atRA hydroxylase, atRA treatment was shown to induce CYP26 mRNA in APL patient derived model cell lines (NB4 and HL–60) (Idres et al., 2005; Ozpolat, Mehta, & Lopez-Berestein, 2005; Ozpolat, Mehta, Tari, & Lopez-Berestein, 2002). Basal CYP26 expression in NB4 cells is very low but mRNA levels can be induced up to 22-fold following 24–96 h of treatment with pharmacologically relevant concentrations of atRA. Furthermore, atRA binding to the RARα nuclear receptor was shown to mediate CYP26 induction (Idres et al., 2005; Ozpolat et al., 2002; Roussel & Lanotte, 2001). These findings support the notion that therapeutic CYP26 inhibition may circumvent APL relapse following atRA treatment by preventing rapid metabolism of atRA due to increased expression of CYP26.

An alternative hypothesis for APL relapse was investigated by Quere et al., in blasts from atRA treatment-naïve APL patients. Blast cells were harvested from patients and treated in vitro with atRA for 3 days. Blasts with delayed maturation, marked as fewer than 50% differentiated cells on day 3, were associated with poor clinical prognosis and categorized as having low-sensitivity to atRA treatment. Blasts with rapid maturation, defined as
greater than 50% differentiated cells following 3 days of *in vitro* incubation with atRA, were associated with significantly improved disease-free survival clinically and therefore categorized as highly sensitive to atRA treatment. In the two classes of blasts, higher transcription of atRA-target genes, transcriptional regulators, and chromatin remodelers was observed in low-sensitive cells. Conversely, CYP26 gene expression was limited to highly sensitive blasts with little to no expression observed in low-sensitive blasts. The authors proposed that low expression of CYP26 in APL blasts with low-sensitivity to atRA *in vitro* led to increased nuclear atRA concentrations and corresponding increased atRA-target gene transcription. Further, when NB4 cells were incubated with either atRA or the CYP26 inhibitor 4-azolyl retinoic acid, accumulation of atRA and development of subclones resistant to atRA-induced differentiation were observed following NB4 cell treatment with 4-azolyl retinoic acid (Quere et al., 2007). While this conflicting data of mechanisms of resistance remain to be resolved, it appears that the activity of CYP26 in regulating nuclear atRA levels is important in preventing APL relapse. Indeed, CYP26-specific RAMBAs have been developed and investigated preclinically but no clinical studies have yet shown the effects of coadministration of atRA and a RAMBA in APL (Li et al., 2013; Njar et al., 2006). Several other strategies have been proposed to address atRA resistance and APL relapse. Intermittent dosing of atRA combined with chemotherapy is used in standard APL therapy during consolidation and maintenance. A significantly lower 2-year relapse rate was reported in patients receiving intermittent atRA compared to patients without atRA treatment during maintenance (Fenaux, Wang, & Degos, 2007).

### 4.2 Neuroblastoma

Neuroblastoma is a malignant tumor comprised of neuroblasts. It develops most commonly in the abdomen but also appears in the neck, chest, and pelvis. Neuroblastoma is the most common extracranial solid tumor in childhood and accounts for 15% of oncology deaths in patients younger than 15 years (Maris, Hogarty, Bagatell, & Cohn, 2007). Treatment of neuroblastoma is based on disease risk stratification and can include surgery alone or chemotherapy and autologous bone marrow transplant. Treatment with 13-*cis*RA significantly improves overall survival of high risk patients when used following transplantation or chemotherapy (Maris et al., 2007; Matthay et al., 1999, 2009). In neuroblastoma cell lines, 13-*cis*RA and atRA have similar potency on cellular differentiation and growth arrest (Reynolds
but 13-\textit{cis}RA is superior to \textit{at}RA in the treatment of neuroblastoma due to its favorable pharmacokinetic and toxicological properties (Reynolds, Matthay, Villablanca, & Maurer, 2003).

Disease relapse is common in neuroblastoma. One approach to improve clinical outcomes is identifying an optimal dosing regimen of 13-\textit{cis}RA. Intermittent high-dose administration of 13-\textit{cis}RA improved clinical outcomes when compared to chronic low-dose administration but significant variability in 13-\textit{cis}RA exposures was still observed causing suboptimal concentrations in some patients (Matthay, 2013; Matthay et al., 2009; Villablanca et al., 1995). Polymorphisms in several enzymes that clear 13-\textit{cis}RA such as CYP2C8, CYP3A5, CYP3A7, and UGT2B1 have been studied as the cause of variability in 13-\textit{cis}RA exposures. 4-oxo-13-\textit{cis}RA is the major 13-\textit{cis}RA metabolite observed in neuroblastoma patients. While 4-oxo-13-\textit{cis}RA exposure was associated with CYP2C8 and CYP3A7 polymorphisms, variability in 13-\textit{cis}RA exposure and 13-\textit{cis}RA:4-oxo-13-\textit{cis}RA ratio could not be attributed to any of the polymorphisms investigated (Veal et al., 2013). It is reasonable to suggest that CYP26 plays a role in the metabolism of 13-\textit{cis}RA since CYP26 is the major catabolic enzyme of \textit{at}RA and isomerization between \textit{at}RA and 13-\textit{cis}RA has been observed (Tsukada et al., 2000).

Given that 13-\textit{cis}RA is generally considered to be devoid of biological activity due to its low affinity for RARs, neuroblastoma cellular differentiation, and growth arrest in response to 13-\textit{cis}RA treatment (Reynolds et al., 2003) can be attributed to isomerization of 13-\textit{cis}RA to \textit{at}RA. However, this has not been directly proven. Determination of retinoid levels following treatment of neuroblastoma cell lines with 13-\textit{cis}RA revealed little extracellular \textit{at}RA but increased intracellular \textit{at}RA due to either intracellular isomerization or selective uptake of \textit{at}RA (Veal, Errington, Redfern, Pearson, & Boddy, 2002). Furthermore, incubation with 13-\textit{cis}RA alone in neuroblastoma cell lines resulted in \textit{at}RA accounting for 15–31% of the total RA. In contrast, after incubation with \textit{at}RA, 13-\textit{cis}RA accounted for <10% of total RA (Armstrong et al., 2007). Intracellular \textit{at}RA concentrations but not 13-\textit{cis}RA concentrations were increased in neuroblastoma cell lines following administration of the CYP26 inhibitor R116010 following either \textit{at}RA or 13-\textit{cis}RA treatment (Armstrong et al., 2005, 2007). This suggests that coadministration of a CYP26 inhibitor may improve clinical outcomes in neuroblastoma patients with low, subtherapeutic 13-\textit{cis}RA concentrations. Surprisingly and in contrast to preclinical results, overexpression of the CYP26A1 gene in neuroblastoma tumors from over 100 patients was found
to be significantly associated with favorable clinical outcomes (Kamei et al., 2009). However, no concentrations of atRA or 13-$\text{cis}$RA were reported in this study. It is possible that higher CYP26A1 expression in the neuroblastoma tumors in clinical samples is indicative of higher RA exposure upon treatment as CYP26A1 is induced by RA. Collectively, these findings highlight the need for more investigation of the concentrations of RA in neuroblastoma and the effects of altered RA exposures to disease outcomes.

5. CONCLUSION

There is considerable evidence that RA plays an important role in modulating innate and adaptive immunity and inflammation, and studies in various disease models suggest that increasing atRA concentrations will be beneficial in inflammatory diseases. The most important role of atRA in the immune system is perhaps inducing T-cell differentiation to Treg cells and suppressing T-cell differentiation to Th17 cells. This activity is also likely important for the anti-inflammatory actions of atRA. In addition, RA has a critical role in inducing the gut homing signals in T-cells. It is not clear how altered expression of RA-synthesizing and -metabolizing enzymes modulates RA concentrations in various immune cells, but there is emerging evidence that CYP26B1 is the predominant enzyme clearing RA in the T- and B-cells and ALDH1A2 appears to be mainly responsible for synthesizing RA in these cells and in DCs. Therefore, alterations in the activity of these enzymes are expected to affect immune homeostasis. However, whether the expression of these enzymes varies dynamically with immune responses and in inflammation is not known. Thus, the endogenous roles of CYP26s and ALDHs in immune modulation are not yet established. It is important to note that endogenous RA is generally synthesized within target cells and RA signaling appears to be modulated by highly specific gradients of RA concentrations (Hogarth et al., 2015). In contrast, when RA is administered exogenously for pharmacological studies, RA gradients are not necessarily generated which may confound the mechanistic information obtained regarding the endogenous regulatory systems and the role of RA in inflammatory processes.

atRA and its stereoisomer 13-$\text{cis}$RA are useful therapeutic agents in the treatment of APL and neuroblastoma, respectively. However, resistance to RA treatment is observed in both diseases and appears to involve induction of the CYP26 enzymes, likely CYP26A1, and increased clearance of RA. Therefore, inhibition of the CYP26s in these cancers is predicted to improve
therapy outcomes. However, some of the mechanistic data of resistance development is controversial and challenging to interpret, as RA induces CYP26 expression but CYP26s also clear RA and likely reduce its concentrations in target cells. Several studies have also suggested that RA signaling and aberrant synthesis and metabolism of RA play a role in other cancers such as breast, prostate, head and neck, colorectal, and ovarian cancers. In many in vitro models of these cancers, increased expression of CYP26A1 appears to promote tumorigenesis while increased concentrations of RA induce apoptotic pathways. Yet, evidence from animal and human studies is incomplete to demonstrate that altered CYP26A1 expression will cause cancer or contribute to cancer development. In addition, it has not been directly shown that RA concentrations are altered in cancer simultaneously with increased CYP26 expression in malignant cells.

Overall, it appears that increasing RA concentrations via inhibition of CYP26 enzymes will have many positive effects in inflammatory diseases such as IBD, psoriasis, and atherosclerosis and in various cancers by promoting apoptosis and cell cycle arrest. However, potent inhibitors of CYP26 enzymes that have suitable in vivo pharmacokinetics to use in disease models or humans have not yet been developed or approved. Therefore, the effect of altered RA homeostasis and the magnitude of required change in RA concentrations to have therapeutic benefit are not well defined. While many of the beneficial effects on the immune system and cancer appear to be associated with increasing RA concentrations, in many other situations, such as fetal development, both too high and too low RA concentrations are detrimental and manifest themselves with similar phenotypes. This may in part explain some of the controversies observed in different inflammatory diseases and cancers and may affect future strategies for development of therapies relating to retinoids.

CONFLICT OF INTEREST
The authors have no conflicts of interest to declare.

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