The Expression of RALDH Enzymes by Small Intestinal Epithelial Cells

IMPLICATIONS FOR THE GUT-ASSOCIATED INNATE IMMUNE SYSTEM

Abstract:

Retinal dehydrogenase (RALDH) is the key enzyme that regulates the production of retinoic acid (RA), an important mediator of immune responses in the gut. RA is the biologically active metabolite of vitamin A. Vitamin A is at the same time processed and metabolized in small intestine. RA produced by intestinal epithelial cells is known to be essential for the induction of characteristic mucosal immune responses by innate immune cells in the intestinal environment. The expression of RALDH enzymes is tissue-specific, with certain tissues expressing higher concentrations than others, and with variations between the different isoforms of the enzyme. RALDH levels and RA production by the innate immune cells in the gut have been shown to be regulated by dietary vitamin A. However the expression of RALDH enzymes in the intestinal epithelium does not decrease with different levels of vitamin A in the diet. In the case of RALDH3 it is even up-regulated in mice fed vitamin A deficient diets. It is believed that this is due to a compensatory mechanism. In this review we look at the genetic and environmental factors that can affect the expression of this enzyme in small intestinal epithelial cells. From this perspective we point at the possible indirect effects that these factors could have on the innate immune cells in the gut through its effects on the production on RA by intestinal epithelial cells.

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Abbreviations

γδT: Gamma-Delta T cell

ADH: Alcohol Dehydrogenase

AhR: Aryl hydrocarbon Receptor

ALDH: Aldehyde Dehydrogenase

APC: Antigen-Presenting Cell

Apo: Apolipoprotein

APRIL: A Proliferation-Inducing Ligand

BAFF: B cell-Activating Factor

BCMO: β-carotene Monooxygenase

CAR: Constitutive Androstane Receptor

C/EBPβ: CCAAT-Enhancer-Binding Protein Beta

CRBP: Cellular Retinol Binding Protein

DC: Dendritic Cell

DGAT: Diacylglycerol Acyltransferase

GADD153: Growth Arrest and DNA Damage Induced Gene 153

GALT: Gut-Associated Lymphoid Tissue

IEC: Intestinal Epithelial Cell

iNKT: invariant Natural Killer T cell

LRAT: Lecithin: Retinol Acetyltransferase

LXR: Liver X Receptor

M cells: Microfold Cells

MLN: Mesenteric Lymph Node

MTP: Microsomal Triglyceride Transfer Protein

NK: Natural Killer cell

NKT: Natural Killer T cell

Nrf2: Nuclear factor (erythroid-derived 2)-like 2

PAMP: Pathogen-Associated Molecular Pattern

PGE_2: Prodtaglandin-E_2

PLRP: Pancreatic Lipase Related Protein

PP: Peyer's Patch

PPAR: Peroxisome Proliferator-Activated Receptor

PTL: Pancreatic Triglyceride Lipase

PXR: Pregnane X Receptor

RA: Retinoic Acid

RAE: Retinoic Acid Early inducible gene

RALDH: Retinaldehyde Dehydrogenase

RAR: Retinoic Acid Receptor

RARE: Retinoic Acid Responsive Element

RBP: Retinol Binding Proteins

REH: Retinyl Ester Hydrolase

ROR: Retinoid-related Orphan Receptor

RXR: Retinoid X Receptor

SDR: Short-chain Dehydrogenase or Reductase

SED: Sub-Epithelial Dome

sIEC: small Intestinal Epithelial Cell

SLPI: Secretory Leukocyte Peptidase Inhibitor

SR-B: Scavenger Receptor Class B

SREBP-1c: Sterol Regulatory Element Binding Protein-1c

Stra6: Stimulated by Retinoic Acid Gene 6

TLR: Toll-Like Receptor

TSLP: Thymic Stromal Lymphopoietin

"How can we remember our ignorance, which our growth requires, when we are using our knowledge all the time?"
Henry David Thoreau

1. Introduction

A. Intestinal Epithelial Cells

The intestinal epithelium is a single layer of polarized cells that separates the lumen of the intestine from the *lamina propia*. On the apical side there are around 10^{14} bacteria (approximately 10 times the number of cells in the human body) (Neish, 2009) and a large amount of food antigens; whereas on the basal side the largest immune organ in the body, the gut-associated lymphoid tissue (GALT), is awaiting (Wershil and Furuta, 2008).

The main characteristic of the mucosal immune response in the gut is therefore a balance between an active suppression of immune responses in the GALT towards the commensal bacteria and food antigens; and the development of appropriate immune responses against pathogenic organisms. The maintenance of this non-responsiveness towards the appropriate antigens is what is currently known as oral tolerance (see Mowat 2003 for a complete review).

However, intestinal epithelial cells (IECs) do not form an impermeable barrier. They are in charge of the uptake of nutrients from the food. Microfold cells (M cells), a subset of IECs, are in charge of antigen uptake, and in consequence are also a main route of entry for bacteria (Magalhaes et al. 2007).

They also have important immune functions: they recognize pathogen-associated molecular patterns (PAMPs), can present antigens directly to T cells and in general contribute to immune homeostasis in the gut by the production of cytokines, chemokines, and the priming of tolerogenic Dendritic Cells (DCs). (See chapter 4, page 15 for further information).

B. Vitamin A

Vitamin A (retinol) is an essential nutrient that is metabolized into retinoic acid (RA). RA has a fundamental role in normal cellular differentiation and proliferation, not only during embryogenesis, but also during post-natal development and adulthood (Niederreither et al 2002, Lin et al. 2003).

The conversion of retinol into RA is controlled by two enzymes. Retinol is firstly converted to retinal by the mediation of alcohol dehydrogenases. Then retinal is irreversibly converted into RA through retinaldehyde dehydrogenases (RALDH). The latter is the rate-limiting step that leads to tissue-specific patterns of RA synthesis. Therefore it is considered the decisive factor for RA production (Niederreither and Dollé, 2008).

Intestinal epithelial stem cells, at the bottom of the intestinal crypts divide every 12 to 16h, proliferating fast. However, when cells approach the top of the crypts and enter the villi they start differentiating (For a complete review see Sancho et al. 2004). Thus, epithelial cells in the small intestine proliferate and differentiate rapidly and can completely renew themselves every 24 to 96 hours (Potten et al. 1992).

RA is known to play a role in the repair of the intestinal epithelium (Katz et al. 2011) and maintaining its barrier function (Osanai et al. 2006). Retinoids have effects on both cell differentiation (Grenier et al. 2007) (De Luca 1991) and to a lesser extent proliferation (Moreb et al. 2008). This is particularly true for epithelial tissue (Thomas et al. 2005). The intestinal epithelial cells in the tips of the villi are in charge of the uptake of retinol (Ong 1993), whereas crypt cells metabolize retinol (probably taken up from the blood) into RA. (Thomas et al. 2005). Consequently, RALDH levels are thought to be higher in crypt cells than in cells that are at the tips of the villi (Thomas et al. 2005). If we hypothesize that RA could be important for the differentiation and proliferation of small intestinal epithelial cells, we could conclude that these cells would require big quantities of RA due to their fast growth and differentiation rate.

C. RALDH Regulation

Dietary vitamin A is required for the expression of RALDH enzymes in both dendritic cells and stromal cells; however it does not modulate the expression of RALDH enzymes in sIECs (Molenaar et al. 2011). BALB/c mice express higher levels and activity of RALDH enzymes in the intestine than C57BL/6 mice. This is translated into higher gut-homing ($\alpha_4\beta_7$) and regulatory molecule (Foxp3) expression in their MLNs, increased lymphocyte levels and IgA secretion in the intestine, maturation of LTi cells and larger secondary lymphoid organs in BALB/c mice (Molenaar 2010). If the differences in RALDH expression are responsible for all these changes in the GALT, it would be useful to know which factors influence RALDH expression in both strains of mice.

It has also been seen that in mice fed conventional chow there is a higher expression of RALDH1 in the proximal part versus the distal part of the intestine. However, in mice fed synthetic diets, where all components are known, the levels of RALDH1 remain low throughout the whole length of the intestine (unpublished data, R. Mebius, personal communication).

This implies that a component of conventional chows is capable of up-regulating RALDH1 levels in the intestine. It has been seen that this component is not dietary vitamin A (Molenaar et al. 2011), but the exact component is not known. Oxysterols have been pointed out to up-regulate RALDH1 in liver cells (Huq et al. 2006), although the effects on sIECs has not been tested.

D. Relation with the Immune System

Both, soluble factors produced by sIECs, and direct contact with sIECs induce the expression of CD103 in DCs. CD103 is a marker for mucosal dendritic cells. One of these soluble factors, RA is known to induce also the expression of RALDH mRNA in DCs. The expression of this enzyme by DCs allows them to produce themselves RA. The production of RA by CD103+ DCs induces the expression of gut-homing proteins on T cells upon their activation and, together with TGF-β, the expression of the transcription factor FoxP3, which is characteristic of regulatory T cells. RA also skews Th responses towards Th_2 responses, it down-regulates Th_{17} responses, and induces gut-homing molecule expression and IgA production by B cells (See Section 2.B, page 18 for more information and references). All these factors point towards an important role of RA produced by sIECs in the modulation of immune homeostasis in the gut.

"What you are shouts so loudly in my ears I cannot hear what you say."
Ralph Waldo Emerson

2. Retinoic Acid

A. Uptake and Storage of Vitamin A

RA is the active metabolite of vitamin A. Dietary forms of vitamin A mainly consist of retinyl esters and β-carotene (Figure 1). The uptake of retinoids from the diet mainly takes place in the proximal part of the small intestine (D'Ambrosio et al. 2011) and is dependent on the amount and type of fat present in the diet (Debier and Larondelle 2005).

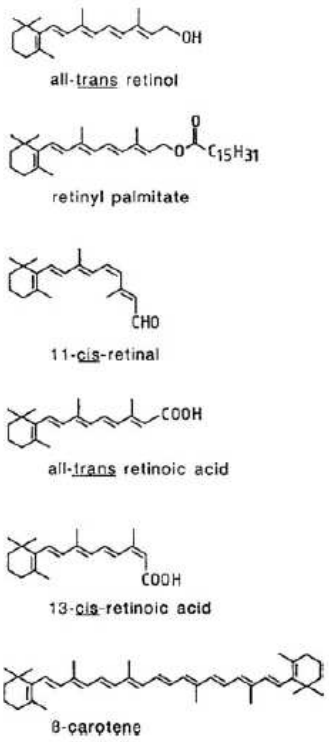


Figure 1: Structural formulas of some naturally occurring retinoids and β-carotene. (Blomhoff and Blomhoff 2006)

Whereas retinyl esters are converted into retinol in the lumen of the intestine by pancreatic enzymes, β-carotene is directly taken up by enterocytes. Already inside the cells it can be shuttled directly to chylomicrons. The other option is, for one particular isoform of β-carotene monooxygenase, to cleave the molecule symmetrically. This way obtaining two molecules of retinal that directly bind to cellular retinol-binding proteins type II (CRBPII). The retinal then is reduced to retinol.

Retinol is taken up from the lumen by enterocytes in the intestinal lining and is immediately bound to CRBPII (Figure 2). Then the retinol present in the cytoplasm is esterified by lecithin: retinol acetyltransferase (LRAT) or by diacylglycerol acyltransferase 1 (DGAT1). Although some of the retinyl esters that are formed remain as lipid droplets in the enterocytes, most of them are transported in chylomicrons, together with other lipids, through the lymphatic vessels to hepatic stellate cells and hepatocytes (Figure 2).

There they are stored until they are required in other parts of the body. In order to mobilize them, the retinyl esters are de-esterified and bound to retinol binding proteins (RBP) to prevent degradation, and are subsequently released into the serum. The discharge of retinol into the serum is tightly regulated and a prolonged deficiency of vitamin A in the diet is required to lower RBP levels in the serum. The RBP-bound retinol is finally

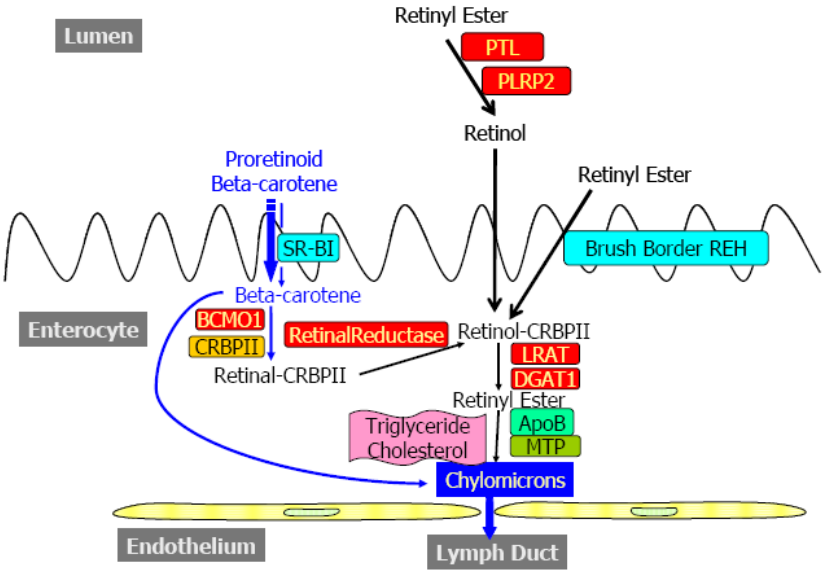


Figure 2: Different routes of uptake and metabolism by small intestinal epithelial cells of the dietary forms of vitamin A (From D'Ambrosio et al. 2011).
Various retinoids are taken up by enterocytes and transformed into retinol. Retinol is esterified and shuttled with other lipids in chylomicrons mainly to the liver, where it is stored.
ApoB: Apolipoprotein B; BCMO: ß-carotene monooxygenase; CRBPII: Cellular retinol binding protein type II; DGAT1: diacylglycerol acyltransferase 1; LRAT: lecithin: retinol acetyltransferase; MTP: microsomal triglyceride transfer protein; PLRP2: pancreatic lipase related protein 2; PTL: pancreatic triglyceride lipase; REH: retinyl ester hydrolase; SR-B1: scavenger receptor class B, type 1

transported to the target cells, in this case small intestinal epithelial cells, where retinol is taken up by a specific receptor: Stra6 (stimulated by retinoic acid gene 6). For a recent extensive review on the uptake and metabolism of vitamin A in the gastrointestinal tract, and its posterior storage in the liver see D'Ambrosio et al. 2011.

B. Importance for the Immune System

Vitamin A, and in consequence its active metabolite, RA, has been shown to be important for splenic natural killer (NK) cell activity in rodents (Ross and Stephensen 1996) and NK and natural killer T cell (NKT) numbers in human peripheral blood (Durianick et al 2010).

Iwata et al. first showed that RA was required for the DC-induced expression of gut-homing receptors on T cells in MLNs and PPs. Later Coombes et al. described that CD103+ DCs induced regulatory T cells via the production of TGF-β and RA. Edele et al. then saw that RA produced by small intestinal epithelial cells was responsible for the expression of gut-homing molecules on T cells. RA production by sIECs also induced RALDH expression in DCs.

Iliev et al. showed that RA was responsible for the induction of the FoxP3 transcription factor in T cells and the down-regulation of Th_{17} responses in a sIEC cell line/bone marrow-derived DC/ CD4+ T cell co-culture. TGF-β was also responsible for the induction of FoxP3 in this same setup. Using a colonic epithelial cell line they also saw an up-regulation of FoxP3 in T cells (only their supernatant was needed) accompanied by an up-regulation of CD103 on bone marrow-derived DCs (this was enhanced by IEC-DC contact). However TGF-β was not responsible for the up-regulation of CD103 on DCs.

At the same time Molenaar et al. showed that MLN stromal cells collaborated with DCs in the induction of $\alpha_4\beta_7$ on T cells. Later on the same group revealed that dietary vitamin A was necessary for the expression of RALDH enzymes in CD103+DCs, but not in sIECs, and that RA itself could induce RALDH expression in CD103+DCs.

C. Enzymatic Regulation

In the target cell retinol is oxidized to retinal by the cytosolic alcohol dehydrogenases (ADH) or enzymes from the short-chain dehydrogenase / reductase family (SDR), the microsomal form (Figure 3). This reaction is reversible and is implicated in the conversion of many other physiologically important alcohols into aldehydes (Duester 2001) and the scavenging of formaldehydes (Westerlund et al. 2007).

However the determinant step is the irreversible conversion of retinal into retinoic acid. This step is catalysed by RALDH enzymes. See Section 3, next page and Figure 3.

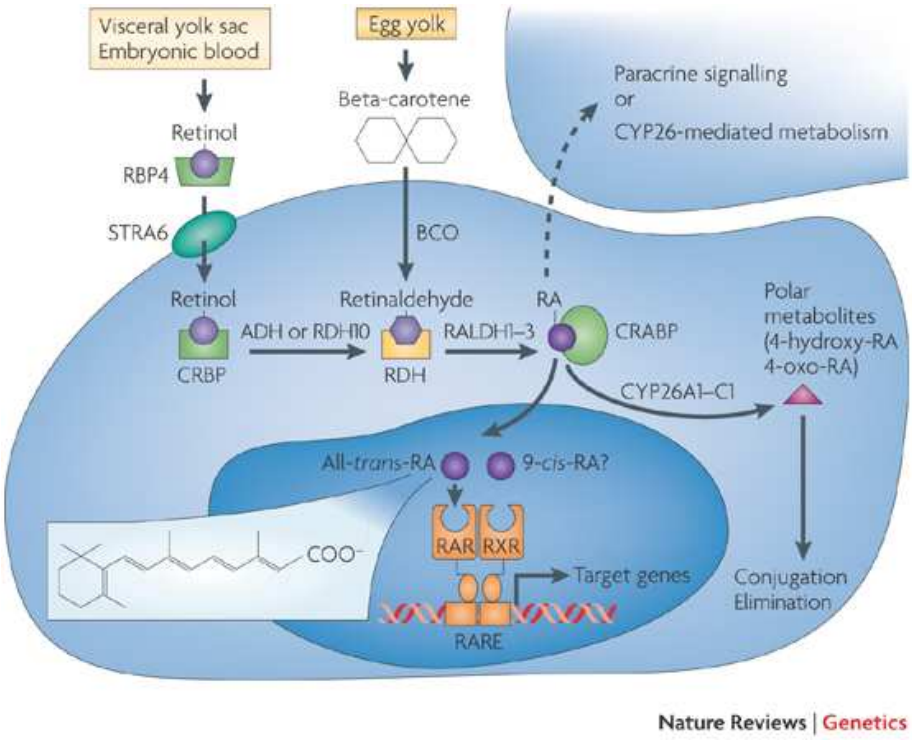


Figure 3: Overview of retinoic acid metabolism in the developing foetus. Taken from Niederreither and Dollé 2008

"All animals are equal, but some animals are more equal than others."
George Orwell

3. Retinal Dehydrogenase

Retinal dehydrogenases (RALDHs) are four enzymes belonging to the aldehyde dehydrogenases (ALDH) group. ALDH enzymes catalyze a number of irreversible oxidations of aldehydes to their carboxylic acids (Alnouti and Klassen 2008). Aldehydes are formed during the metabolism of different endogenous and exogenous compounds and are highly reactive. Therefore ALDHs are considered as detoxifying enzymes. They also participate in the metabolism of alcohols, biogenic amines, vitamins, steroids and lipids (Elizondo et al. 2009).

There are four main variants of RALDHs. RALDH1, or Aldh1a1, is a tissue-specific enzyme in charge of the synthesis of RA, but also detoxifying aldehyde products of lipid peroxidation. RALDH2, or Aldh1a2, has the highest substrate specificity for retinal. It is also tissue-specific and is highly involved in tissue development during embryogenesis. RALDH1 and RALDH3 become more important during the last phases of embryo development. RALDH3, or Aldh1a3, is thought to be the constitutive isoform of RALDHs, as it is expressed in low quantities in almost all tissues. RALDH4, or Aldh8a1, is the only member of the RALDH enzymes known to prefer 9-cis-retinal to all-trans-retinal as a substrate (Lin et al. 2003).

A. Localisation in the Intestine

In 1998 Bhat studied for the first time the distribution of RALDH1 enzymes in the epithelium of the gastrointestinal tract of the rat. They had previously seen that it was in the epithelium of these tissues were the highest expression of these enzymes was localised.

In this study, they observed RALDH1 mRNA to be expressed mostly in the rat small intestinal epithelium before birth, whereas expression decreased after birth. This coincided with a lower expression of the protein at birth. They also observed a down-regulation of the expression of RALDH1 mRNA in the small intestinal epithelium if vitamin A deficient rats were given vitamin A. This is in contrast with the results obtained by Molenaar et al. 2011 who did not observe any changes in RALDH1 mRNA in the small intestine of mice with different quantities of vitamin A in the diet.

In 2002 Niederreither et al. looked at the expression of RALDH enzymes in developing mice embryos. RALDH2 was expressed from days 14.5 to 16.5 in mesenchymal cells of the

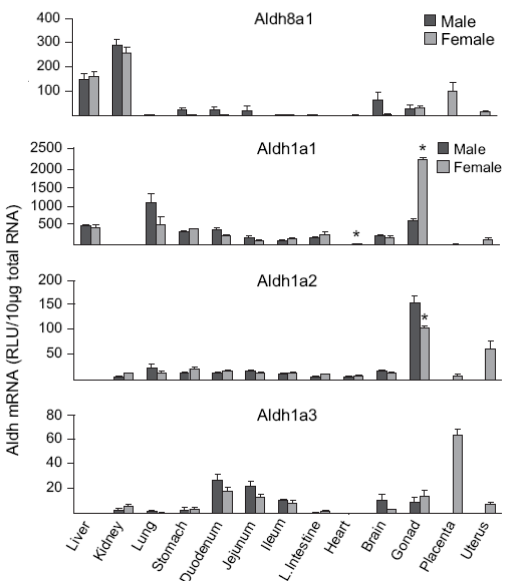


*Figure 4: Total RNA was isolated from approximately 8-week-old male and female mice and analyzed by the bDNA signal amplification assay for Aldh8a1, 1a2, 1a3, and 1a1 mRNA expression. The data are presented as mean RLU ± SEM (n = 5). *Represents a statistically significant difference (p≤0.05) between males and females. Alnouti and Klassen, 2008*

stomach and intestine. RALDH3 then appeared on days 14.5 to 16.5 in the lamina propia. Whereas finally, RALDH1 appeared on day 16.5 in the epithelium, then at birth, it moved to the base of the intestinal glands and, when the intestine differentiated, the enzyme appeared in the stems of the intestinal villi. The changes in the expression of the enzymes point towards the different roles played by the three enzymes at different stages of development.

In 2007 Westerlund et al. described how the expression of RALDHs in the small intestine both in rat and mice was restricted to the epithelium at the base of the villi. Only in the ileum of rats, the expression of RALDH enzymes decreased.

However in 2008 an interesting paper (Alnouti and Klassen) was published showing thoroughly the expression of mRNA for the different ALDHs described up until then. They saw a high expression of RALDH1 in the small intestine, together with lower levels of RALDH2, 3 and 4. They all presented higher levels in the duodenum, decreasing towards the distal end of the intestine (Figure 4, take into account the different scales used for each enzyme).

B. Genetic Regulation

Little is known about how RALDH genes are regulated. However there is evidence that different genetic backgrounds in mice influence RALDH mRNA expression in the intestine (Molenaar 2009), indicating a genetic regulation.

The aryl hydrocarbon receptor (AhR) is highly expressed in sIECs (Chmill et al. 2010). AhR is a transcription factor, normally inactive, that is involved in development of the immune system but is mainly known for its role in chemical sensing. The ligation of AhR induces the activation of the “AhR gene battery”, related mostly to detoxifying enzymes (Nebert et al 1993).

In the liver of AhR-null mice, RALDH1 mRNA was down-regulated, whereas retinoic acid α receptor (RARα) mRNA levels were increased, if compared to wild type mice. In this study they suggest that RA concentration could be a key factor, through various intermediates, for the genetic regulation of RALDH (Figure 5) (Elizondo et al. 2009). However direct AhR ligation (with 2,3,7,8-tetrachlorodibenzo-p-dioxin, polychlorinated biphenyl 126 or β-naphthoflavone) in the liver of C57BL/6 mice does not affect the expression of RALDH-1, 2 or 3 mRNA (Alnouti and Klassen, 2008). This data points towards an indirect regulation of RA concentration by AhR through its breakdown by CYP enzymes (Elizondo et al. 2009) and an indirect regulation of RALDH-1 levels by AhR.

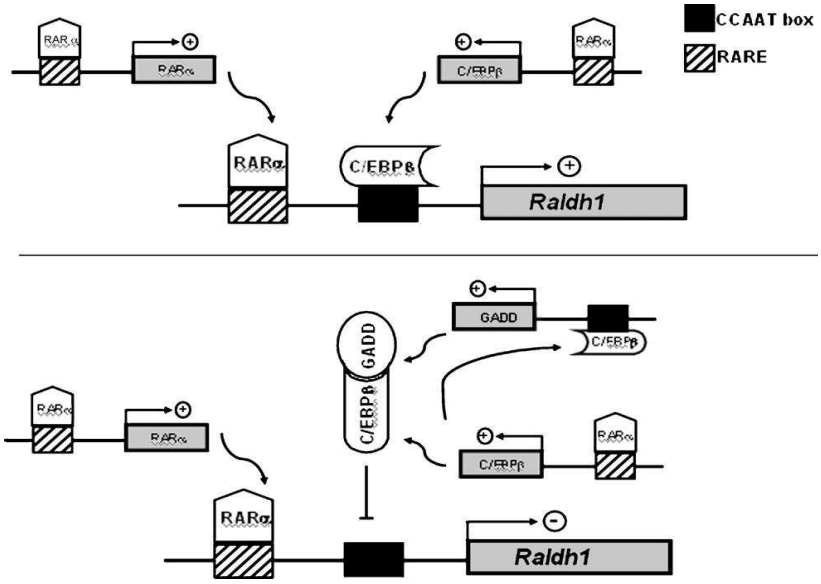


Figure 5: Model of autoregulation of the RALDH1 gene promoter by heterodimerization of GADD153 and C/EBPβ. At low RA concentrations, RARa and C/EBPβ transactivate the RALDH1 promoter (top panel). When RA levels increase, RARa transactivate the C/EBPβ promoter increasing the C/EBPβ abundance resulting in an increase of GADD153 amount and the formation of GADD153– C/EBPβ heterodimers (lower panel). (Elizondo et al. 2009)

Despite this, Alnouti and Klassen did show that various xenobiotics could induce the expression of RALDH1 and RALDH4 in the liver of mice (See Table 1 in Environmental Regulation). These compounds included various constitutive androstane receptor (CAR) activators, pregnane X receptor (PXR) ligands (also in liver and the small intestine of rats, Hartley et al. 2004), peroxisome proliferator-activated receptor α (PPARα) ligands and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activators.

The constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) are involved in the sensing of endobiotic and xenobiotic lipophilic toxic compounds, and the promotion of the expression of detoxifying enzymes. They appear both in the liver and in the small intestine of mice and can form heterodimers with RXR (Maglich et al. 2002). CAR interacts with PPARα (see below) and PPARγ coactivator-1α. (Xiao et al. 2010)

Some of their ligands (see Environmental Regulation) have been shown to up-regulate RALDH1 and 4 in the liver (Alnouti and Klassen, 2008), whereas the effect on the small intestine is not so clear (Maglich et al. 2002). CAR is also particularly involved in the regulation of bilirubin metabolism (Xiao et al. 2010), a downstream process of cholesterol metabolism.

Peroxisome-proliferator alpha (PPARα) is a regulator of lipid metabolism in the liver and promotes the uptake, use and catabolism of fatty acids. As CAR and PXR, PPARα forms heterodimers with RXR (Fruchart 2009). It is also thought to induce detoxifying enzymes through the crosstalk with other transcription factors (Motojima and Hirai 2006).

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a master regulator of antioxidant responses, in particular to oxidative conditions caused by xenobiotics. It is specifically capable of inducing a different set of enzymes distinct of CYP enzymes: the glucuronosyltransferases, phase II enzymes (Niture et al. 2010).

Liver X receptors (LXRα and LXRβ) sense a particular kind of stress: cholesterol overload. If they are activated, they promote the reverse cholesterol transport from the peripheral tissues to the liver (Xiao et al. 2010). The knockdown of LXRs and sterol regulatory element binding protein-1c (SREBP-1c) decreased levels of RALDH1 and 2 enzymes in the liver. This decrease was rescued with the supplementation of SREBP-1c, showing this protein is a mediator of the regulation of RALDH by LXR (Huq et al. 2006). LXRα is also expressed in high levels in the small intestine, whereas LXRβ is expressed ubiquitously, indicating that LXRα is the most likely candidate if the effect can be extrapolated to sIECs. LXR has also been linked to the conversion of cholesterol in the liver into bile acids (Zhao and Dahlman-Wright, 2010).

In addition, LXR has been shown to interact with CAR (see above) and crosstalk with retinoid-related orphan receptors (RORs). RORs are related to regulation of development, metabolism, and immune function. LXR and RORα seem to suppress each other mutually (Xiao et al. 2010). Furthermore, 7 α-hydroxycholesterol, an intermediate of bile acid metabolism and an LXR ligand, modulates RORα and RORγ activity in hepatocytes (Wang et al. 2010).

The transcription factors that have been seen, directly or indirectly, to affect RALDH enzymes are involved in detoxification mechanisms, but are also quite related to lipid and

fatty acid metabolism. This is coherent with its function as a detoxifier of the aldehyde and alcohol precursors of RA, but also with the lipophilic nature of RA.

The relevance of the exogenous ligands of these transcription factors will be studied in detail in the next section: Environmental Regulation.

C. Environmental Regulation

1. Dietary Vitamin A

The regulation of RALDH levels by vitamin A or its derivatives has been suggested previously (Jaensson-Gyllenbäck et al 2011) (Elizondo et al. 2009). This mechanism of RALDH regulation makes sense in the liver where RA levels must be kept under control and tightly regulated. However, this is not the case in sIECs, where there are large amounts of retinoids processed in steady state conditions. Admittedly, RALDH regulation by RA could be logical in the context that RA in sIECs might be involved in other functions of these cells, especially related to cholesterol absorption and removal (Grenier et al. 2007).

However, in Molenaar et al. 2011 dietary vitamin A does not seem to regulate the expression of RALDH enzymes in murine small intestines. In their vitamin A deficient animals, both serum levels and storage in the liver of RA were low. However, these animals did not present lower levels of RALDH 1, 2 or 3. This means that it does not ultimately depend on the levels of RA available for these cells, whether the RA comes from the bile (Jaensson-Gyllenbäck et al 2011) or the serum.

2. Other Environmental Influences

Other dietary components that can affect RALDH expression in the small intestine are unknown. However, it is useful to look at the ligands for the transcription factors studied in the previous section: Genetic Regulation.

If we look at Table 1, we can see the ligands that showed an effect on RALDH1 or 4 in the liver in Alnouti and Klassen, 2008. Although some of them are synthetic and unlikely to be present in animal feed (e.g. Phenobarbital), others could be present due to their natural presence in food (diallyl sulphide) or to their expected addition as preservatives (ethoxyquin and butylated hydroxyanisole). Phthalates, although synthetic, could be used in storage plastic containers and pollute the food. In addition, different herbicides (clofibric acid) and other environmental pollutants could be present in the food.

Target Transcription Factor	Compound	Use	Known Effects
CAR	1,4-bis[2-(3,5-dichloropuridyloxy)]benzene	Synthetic CAR agonist of the Phenobarbital family	CYP450 inducer (Smith et al. 1993)
	Diallyl sulfide	Natural component of garlic, onions and leeks that gives them taste and smell (Lawson et al. 1991)	It is a well known detoxicant (Chen et al. 2004), it has antimicrobial properties (O'Gara et al. 2000) and has been shown to be beneficial for colorectal cancer (Wargovich 1987).
	Phenobarbital	Anticonvulsant drug	CYP450 inducer (Ueda et al. 2002) and inducer of bilirubin conjugation (Robinson et al. 1971),
PXR	Pregnenolone-16a-carbonitrile	Steroid derivative	CYP450 inducer (Dalvi et al. 2002)
	Spironolactone	Aldosterone lowering drug, diuretic	Increased urine secretion, increase of duodenal bleeding (Russo et al. 2008)
PPARα	Clofibric acid	Herbicide	Cholesterol lowering drug (Abourbih et al. 2009)
	Ciprofibrate		Cholesterol lowering drug (Rizzo and Berneis 2007)
	Di-(2-ethylhexyl) phthalate	Plasticizer	Hepatotoxic, inducer of liver cancer, inducer of oxidative stress and endocrine disrupter (ATSDR 2002)
Nrf2	Oltipraz	Antischimatosis and antitumour drug	Antioxidant (Choi et al. 2010) and chemopreventive (Clapper 1998)
	Ethoxyquin	Pesticide and food preservative	Genotoxic and antioxidant (Błaszczyk et al. 2006)
	Butylated hydroxyanisole	Food preservative	Antioxidant and possible inducer of cell proliferation and oxidative damage (Iverson 1999)

Table 1: Tested chemical compounds that modify RALDH1 or 4 in C57BL/6 mice, sorted by their target transcription factor. Modified from Alnouti and Klassen 2008

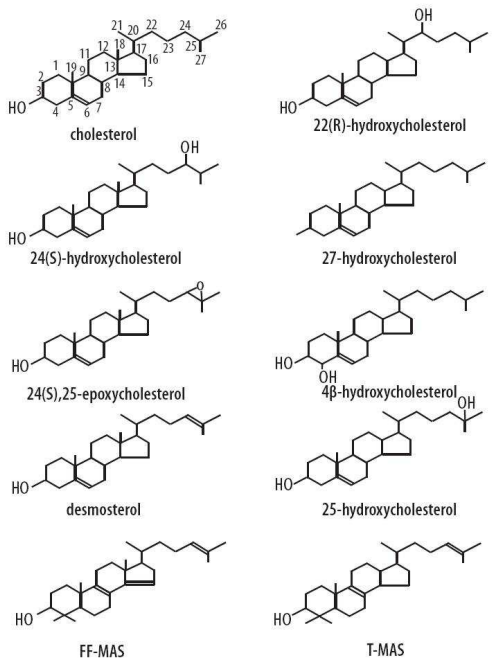


Figure 6: Most important endogenous sterol agonists of LXR. FF-MAS – follicular fluid meiosis-activating sterol, T-MAS – testes meiosis-activating sterol. Wójcicka et al. 2007

In 2006 oxysterols, oxidative derivatives of cholesterol, where shown to up-regulate the expression of RALDH1 and 2 in the liver (Huq et al. 2006). Oxysterols are natural ligands of LXR (See Figure 6) (Wójcicka et al. 2007). This implies that these compounds found both in the diet and endogenously, could act upon the expression of RALDH enzymes in the small intestine.

What many of these compounds have in common is their relation to cholesterol and/or other steroids. The different steroidal hormones derive from cholesterol. During their synthesis and catabolism, which occur mainly in the liver, they require a number of CYP enzymes. Steroids are

degraded and eliminated into the bile together with cholesterol, bile pigments (such as bilirubin) and retinoids. All this links vitamin A and sterol metabolism in the liver, and suggests that these results could not be applied to the small intestine.

On the other hand, vitamin A is picked up by the intestine, transferred to chylomicrons and transported to the liver with other lipids, including a number of sterols (See section 2.A.). In addition, certain transcription factors have both effects on the liver and the small intestine (For a full review see Chiang 2002). Therefore, some phases of vitamin A and sterol metabolism could potentially be linked in both the liver and sIECs.

It is also possible that the effects of these compounds are the opposite in both tissues. The mechanisms to prevent excess cholesterol promote its shuttling to the liver and its excretion via the bile through the reverse cholesterol transport pathway (Kruit et al. 2005) (Xiao et al. 2010).

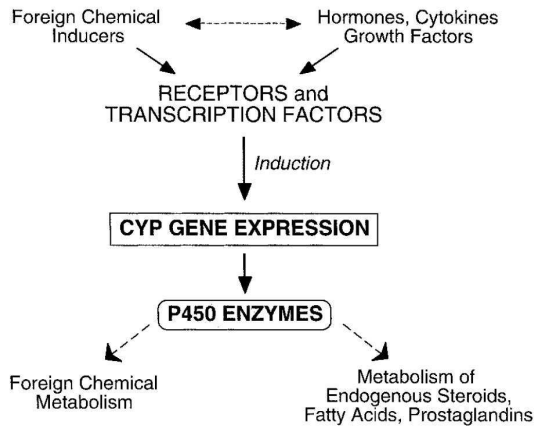


Figure 7: CYP gene induction: Cross-talk between foreign chemical and endogenous regulator pathways (Waxman 1999)

What is clear is that there are numerous crosstalks between the different nuclear receptors, hormones, growth factors, cytokines and xenobiotics (Figure 7). Recently there has been a breakthrough in the research between these interactions and the specificity and interaction of the different xenobiotic on these transcription factors using microarrays. An enlightening review was done in 2007 by Woods et al. (Figure 8). In this review, it is clear how complex the relations between the transcription factors are, but also the wide arrange of enzymes, proteins, cytokines and other small molecules that affect the different pathways.

From a completely different perspective, the RALDH1 gene has also been shown to be able to respond to interleukin-6 in human liver tissue, pancreas tissue, hepatoma cells and genital skin fybroblasts (Yanagawa et al. 1995). This has also been seen in prostate cancer cells, both by exposure and inhibition of IL-6 (Hellsten et al. 2011). C/EBPβ is also referred to as nuclear factor for IL-6 expression, and is an important regulator of IL-6 production. As Elizondo et al. suggest (see Figure 5) C/EBPβ induces RALDH1 expression in the liver. This could indicate a response of the cells to early inflammatory responses by up-regulating RALDH1.

This phenomenon has not been looked into further detail, but RALDH enzymes have been seen to be up regulated in cancer cells, and have been proposed as functional markers of cancer stem and progenitor cells (Douville et al. 2009). This could be a side-efffect of the increased production of IL-6 seen in certain cancers (Schafer and Brugge 2007) (Atreya and Neurath 2005).

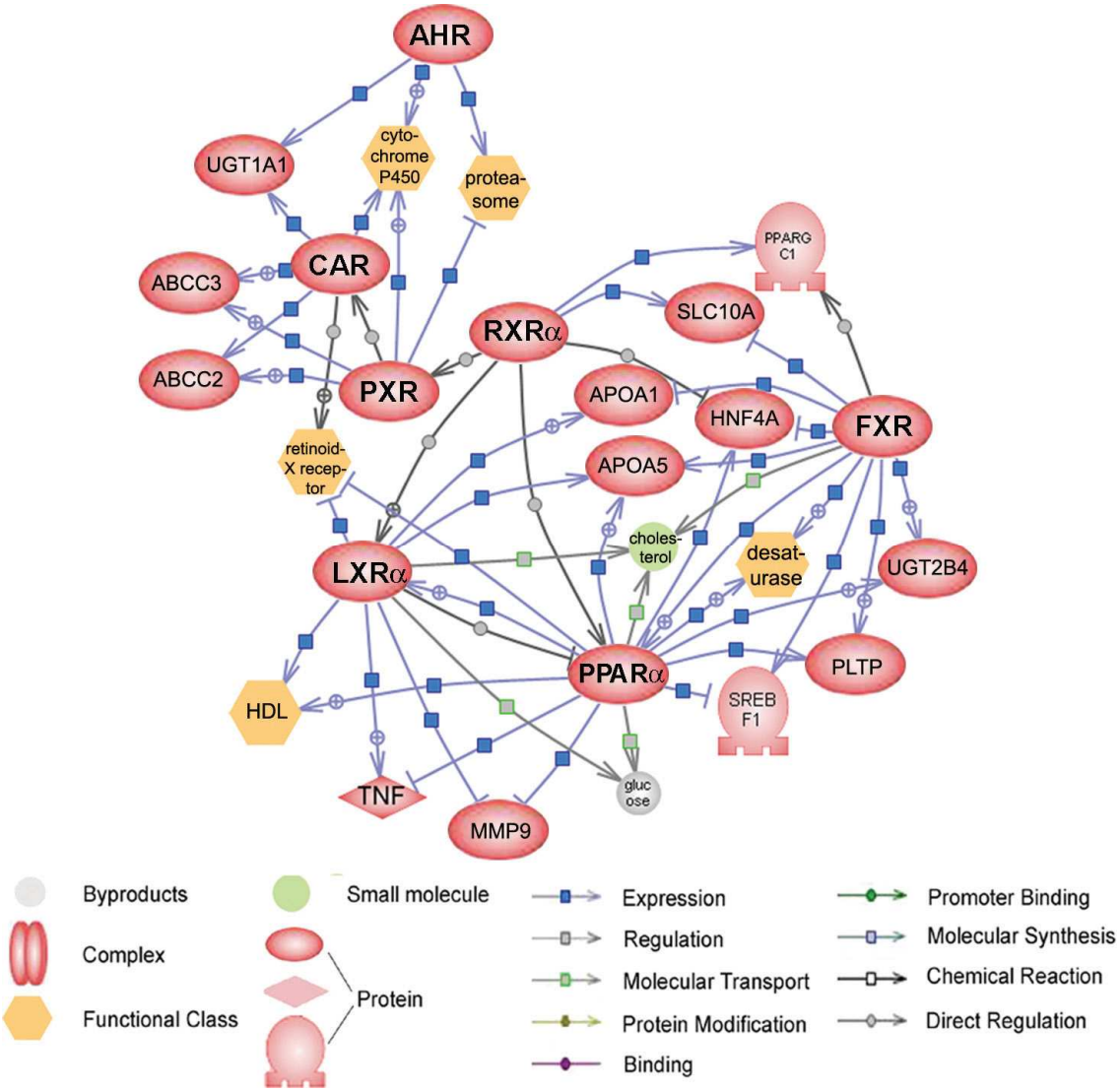


Figure 8: Crosstalk and co-regulation among nuclear receptors. Designation of nodes and edges is indicated at the bottom of the figure (Woods et al. 2007)

"...Before I built a wall I'd ask to know what I was walling in or walling out, and to whom I was like to give offence. Something there is that doesn't love a wall, that wants it down. ..."
Robert Lee Frost

4. Small Intestinal Epithelial Cells

Small intestinal epithelial cells (sIECs) form a single cell layer. They are tightly stacked together due to the tight junction proteins that connect them to each other. This enables the barrier to act as a selective filter, allowing only the uptake of certain molecules, and keeping other luminal contents out. However, sIECs do not only serve as a barrier, but also as a connection between the external world and the immune system in the gut (Figure 9).

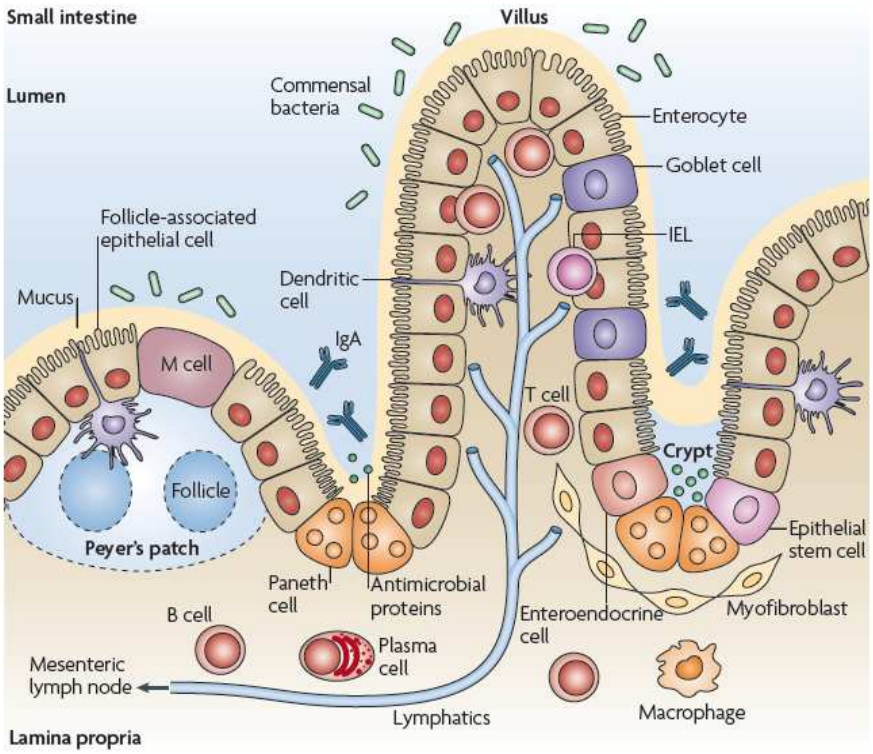


Figure 9: Anatomy of the Intestinal Immune System, Abreu 2010

The epithelial cells define the limit between the external world and the body of the host. A single layer of enterocytes is the main component of this barrier. In between them lay stem cells that regenerate the epithelial tissue. Goblet cells secrete the mucus that protects the lining of the villi form the commensal bacteria. Enteroendocrine cells secrete different hormones. Paneth cells secrete antimicrobial proteins into the lumen of the intestine. The epithelial cells that cover Peyer's patches (the follicle-associated epithelial cells) have distinct innate immune signalling patterns. They can differentiate into M cells that facilitate the passage of antigens into the subepithelial domes of the Peyer's patches. Different subsets of macrophages and dendritic cells in the lamina propia are ready to respond to the presence of an antigen in different ways, deciding what kind of immune response (or tolerance) is required.

sIECs are able to communicate with this external world. PAMPS are distinct invariant molecular patterns that are associated with different types of microbiota, e.g: peptidoglycan and lipopolysaccharide for bacteria, βglucans for fungi, double-stranded ribonucleic acids for viruses and phosphoglycan for parasites. Various cell types in the immune system are able of recognizing these PAMPs through pattern recognition receptors, of which the most important are toll-like receptors (TLRs) (For a full review of how PAMPs are recognized in the intestine see Magalhaes et al. 2007.). sIECs express TLRs on both their basal and apical membranes and can therefore recognize PAMPs (Cerovic et al. 2009). TLR ligation in IECs is implicated in promoting IEC proliferation, secreting IgA into the lumen, the maintenance of tight junctions and the expression of antimicrobial peptides (Abreu 2010). See Figure 9.

IECs are known to be fundamental for the maintenance of oral tolerance and, under steady-state circumstances, induce tolerant responses towards commensal bacteria. They are not only capable of taking up antigen and passing it on to professional antigen presenting cells (APCs), they are also capable of acting themselves as non-professional APCs as they expresses MHC class II constitutively (Chehade and Mayer, 2005). In contrast with professional APCs, sIECs induce a suppressor phenotype in the antigen-specific T cells, regulated through the expression of C1d (Bland and Warren 1986) and gp180 (Yio and Mayer 1997). However, they are also able to recognize and respond to pathogenic bacteria by producing pro-inflammatory cytokines such as MIP-2 in mice (IL-8 in humans), IL-6, IL-12 and TNF-α (See Figure 11). Commensal bacteria, however, are able to regulate pro-inflammatory and anti-inflammatory cytokines mainly through the production of short-chain fatty acids (Magalhaes et al. 2007).

There are mainly two theories as to how sIECs can distinguish between stimulation by commensal and pathogenic bacteria. Interestingly it has been shown that IECs respond differently to toll-like receptor-9 (TLR9) stimulated on the apical or basal side. Apical stimulation of sIECs causes mainly tolerogenic responses, whereas basal stimulation will produce pro-inflammatory molecules (Lee et al. 2006). This would favour the hypothesis that commensal bacteria living in the lumen induce tolerogenic responses, whereas pathogenic bacteria that possess invasive factors, and are therefore more likely to reach the basal membrane, or bacteria that have invaded the *lamina propia* after intestinal injury, promote immune responses.

However, it has been seen that apical stimulation of TLR3 can also induce immune responses (Zhou et al. 2007). This points towards a second theory that states that epithelial cells on the tip of the villi, and more prone to contact with commensal bacteria, promote tolerogenic responses, and that stimulation of cells in the crypt, a more sterile environment, would indicate a pathogenic invasion, and therefore promote an immune response (Rescgino et al. 2008).

A. RALDH Expression in sIECs

The hypothesis of the distribution of tolerogenic responses vs. immune responses along the axis of the villi stated above interestingly contradicts the distribution of RALDH enzymes seen by Thomas et al. (Figure 10). In this article, they see that cells on the tip of the villi

express less RALDH enzymes, than those in the crypt area, and will therefore produce less RA. However, this distribution could be more related to the need for cell differentiation in the intestinal crypts, as RA is thought to be required for this process in sIECs (Thomas et al. 2005).

If the epithelial cells at the top of the villi were responsible for more tolerogenic responses to bacteria in the gut, they would express higher amounts of RALDH, to produce more RA.

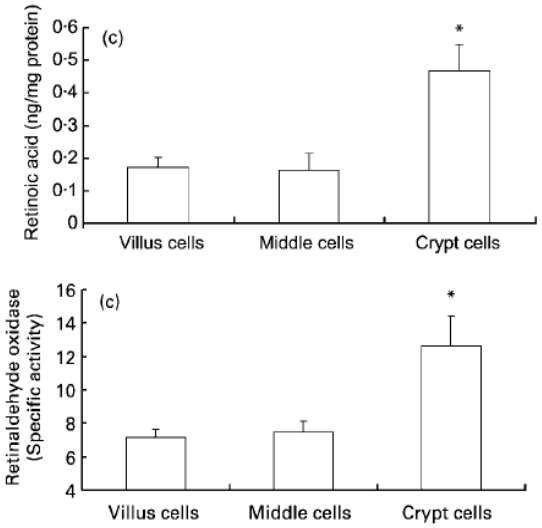


*Figure 10: RA and RALDH expression in enterocytes from untreated rat intestine. Each value represents the mean with standard deviation represented by a vertical bar of six separate experiments (n 6) with duplicate estimations. *P<0·05 compared with villus cells. (Thomas et al. 2005)*

A recent paper, Merlos-Suárez et al. 2011, contradicts this distribution of RALDH enzymes along the tip-crypt axis. They sorted primary mouse intestinal epithelial cells depending on their expression of a certain stem cell marker gene (EphB2). They performed microarrays on these cells and characterized them.

They discovered that aldh1a1 was a gene characteristic of the so-called "Late TA" cells. These cells correspond to the more differentiated epithelial cells, which are towards that top of the villi. They saw that these cells expressed this gene 2.66 times more than the intermediate cells. The difference was even higher between intermediate and crypt cells (4.12 times).

This again would point towards the influence of RA on the distribution of immune versus tolerogenic responses along the crypt-tip axis (Rescigno et al. 2008).

B. RALDH Influence on Cytokine/Chemokine Expression by sIECs

IECs can produce a wide variety of cytokines and chemokines. Some of them can potentially affect the maturation and differentiation of DCs: GMCSF, IL-6, IL-10, IL-12, CCL9, CCL20, TGF-β, and others (See figures 11 and 13).

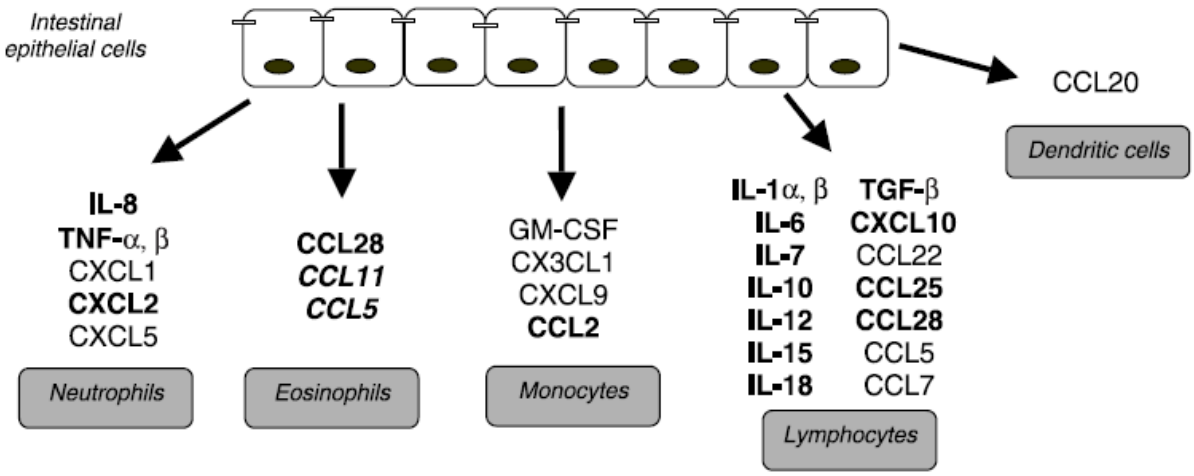


Figure 11: Summary of the cytokines and chemokines produced by intestinal epithelial cells. The usual target of each cytokine is shown although in some cases the same cytokine may act on more than one cell type. The figure includes constitutive and induced cytokine. IL: interleukin CCL/CXCL: Chemokine (C-C/C-X-C motif) ligand; TGF: transforming growth factor. Oswald 2006

RA has been shown to affect the maturation and differentiation of DCs, and IL-6 has been shown to affect levels of RALDH in human liver and pancreas tissue, but not the other way round (See section 3.C.2). It would be interesting to see if the cytokine production profile of sIECs is connected to their expression of RALDH enzymes.

"We are what we repeatedly do. Excellence, then, is not an act, but a habit." Aristotle

5. The Gut-Associated Lymphoid Tissue

The gut-associated lymphoid tissue (GALT) is in charge of either allowing or silencing immune responses towards non-self-antigens that are in the gut lumen: food or commensal bacteria versus pathogenic bacteria. When pathogens are recognized, immune responses are induced, whereas in the steady state situation tolerance towards food and commensal antigens is maintained.

The GALT is comprised of:

- The IECs, a single-cell barrier, these cells adhere tightly together

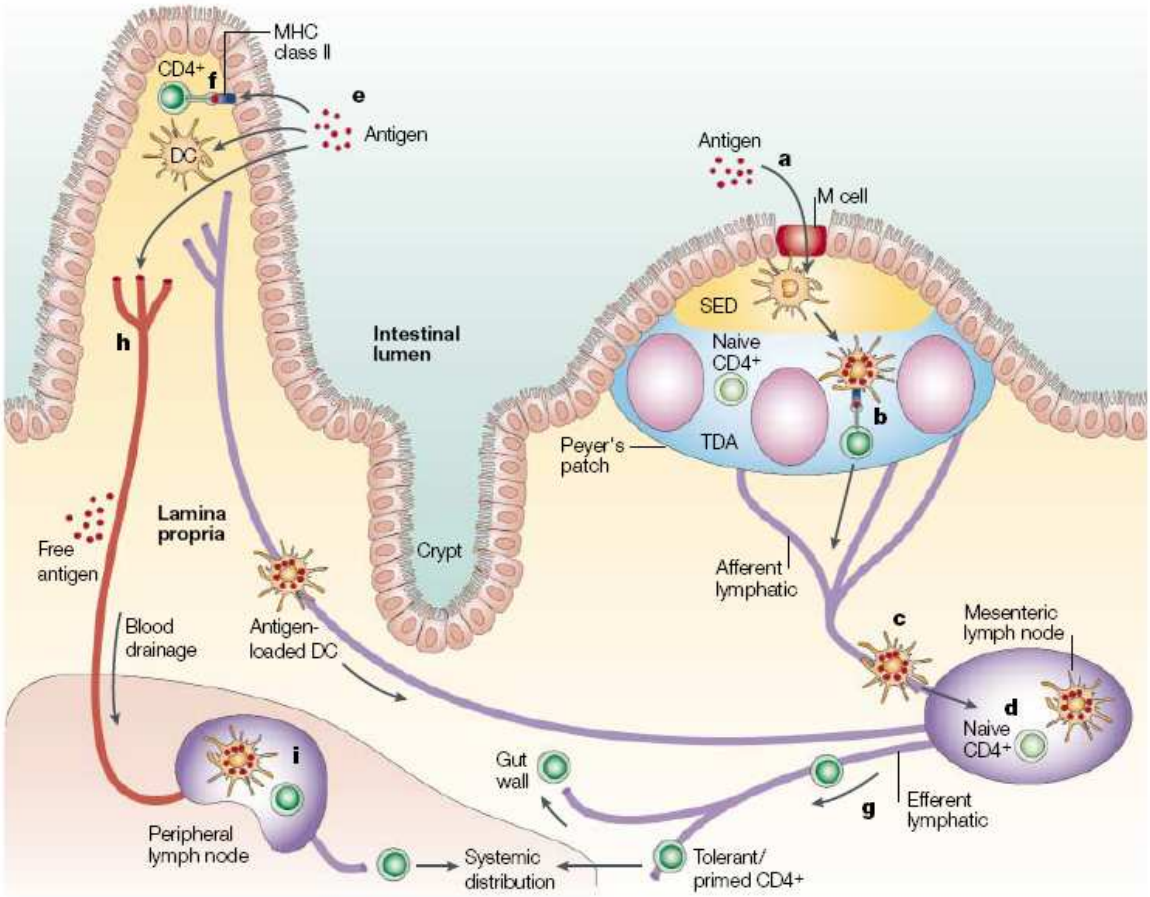


Figure 12: Antigen Uptake and Recognition in the Gut-Associated Lymphoid Tissue (Mowat 2003)
Antigen might enter through the microfold (M) cells in the follicle-associated epithelium (FAE) (a), and after transfer to local dendritic cells (DCs), might then be presented directly to T cells in the Peyer's patch (b). Alternatively, antigen or antigen-loaded DCs from the Peyer's patch might gain access to draining lymph (c), with subsequent T-cell recognition in the mesenteric lymph nodes (MLNs) (d). A similar process of antigen or antigen-presenting cell (APC) dissemination to MLNs might occur if antigen enters through the epithelium covering the villus lamina propia (e), but in this case, there is the further possibility that MHC class II+ enterocytes might act as local APCs (f). In all cases, the antigen-responsive CD4+ T cells acquire expression of the α4β7 integrin and the chemokine receptor CCR9; leave the MLN in the efferent lymph (g) and after entering the bloodstream through the thoracic duct, exit into the mucosa through vessels in the lamina propia. T cells which have recognized antigen first in the MLN might also disseminate from the bloodstream throughout the peripheral immune system. Antigen might also gain direct access to the bloodstream from the gut (h) and interact with T cells in peripheral lymphoid tissues (i).

- The different organized lymphoid tissues: Peyer's patches, isolated lymphoid follicles and MLN
- The immune cells: such as DCs, macrophages, B cells and T cells

In between the epithelial cells and adjoining the PPs, lie the M-cells. They facilitate the transport of antigens into the gut for the DCs to recognize. The DCs either present them to the T-cells directly in the PPs or they take them to MLN and present them there.

A small percentage of antigen can also be taken up directly through MHCII+ epithelial cells or by direct uptake via DCs, and transported to the MLN. When it reaches the MLN specific-antigen responsive T- cells are distributed back to the gut and into the system, where they will recognize and tolerate the antigens when presented again. There is always the possibility that free antigen reaches the bloodstream and interacts with T-cells in the peripheral lymphoid tissues. (Figure 12)

This complex system of antigen recognition allows the GALT to maintain tolerance towards normal antigens that are present constantly in the gut.

A. The Innate Immune Cells in the GALT

In the GALT a variety of immune cells are present. The innate immune cells are there to sample the gut constantly and detect both the signals of infection and stress. They can then interact with the adaptive immune cells to elicit more complex responses and "memorize" them, in the case of a second encounter with the antigen. In the GALT there are two main cells that belong to the innate immune system and are intrinsically linked to oral tolerance: dendritic cells and macrophages (Coombes and Powrie, 2008). There are also two types of cells that have both characteristics of innate and adaptive immune cells, and are especially interesting due to the plasticity of their reactions and their relationship with RA: invariant natural killer T cells (iNKT) and gamma-delta T cells (γδT).

1. Phagocytes

Dendritic cells (DCs) are the main antigen-presenting cells (APC) in the GALT, together with macrophages (Coombes and Powrie 2008). Nevertheless, compared to these, they have the added ability of activating naïve T cells. (Cerovic et al. 2009) They are responsible for the sampling of the lumen for antigen, recognising the antigen gathered by M cells, epithelial cells or by themselves, processing it, and presenting it to T cells (Chehade and Mayer 2005) (Mowat 2003). DCs integrate all the local peripheral environmental condition

signals and direct the development of the immune response. This makes DCs the communication system between the peripheral tissue and secondary lymphoid tissues and also the main link between the innate and adaptive immune systems (Cerovic et al. 2009).

Macrophages are the second type of APCs that appear in the intestinal lamina propia. They are thought to help out in the antigen recognition and tolerance induction of the intestinal environment, whilst keeping their bactericidal activity (Rescigno et al. 2009).

There is a special subset of CX3CR1+CD11b+ phagocytic cells (Varol et al. 2010) that is in close contact with IECs. These cells are able to fit dendrites in between the tight junctions of IECs. It has been shown that TLR stimulation within sIECs is important to provoke this process (Rescigno et al. 2009).

A subtype of CD11b+F4/80+ macrophages is known to phagocytise and eradicate bacteria but is refractory to TLR stimulation; it also promotes colonic wound healing. TREM2+ macrophages can promote colonic epithelial regeneration (Varol et al. 2010).

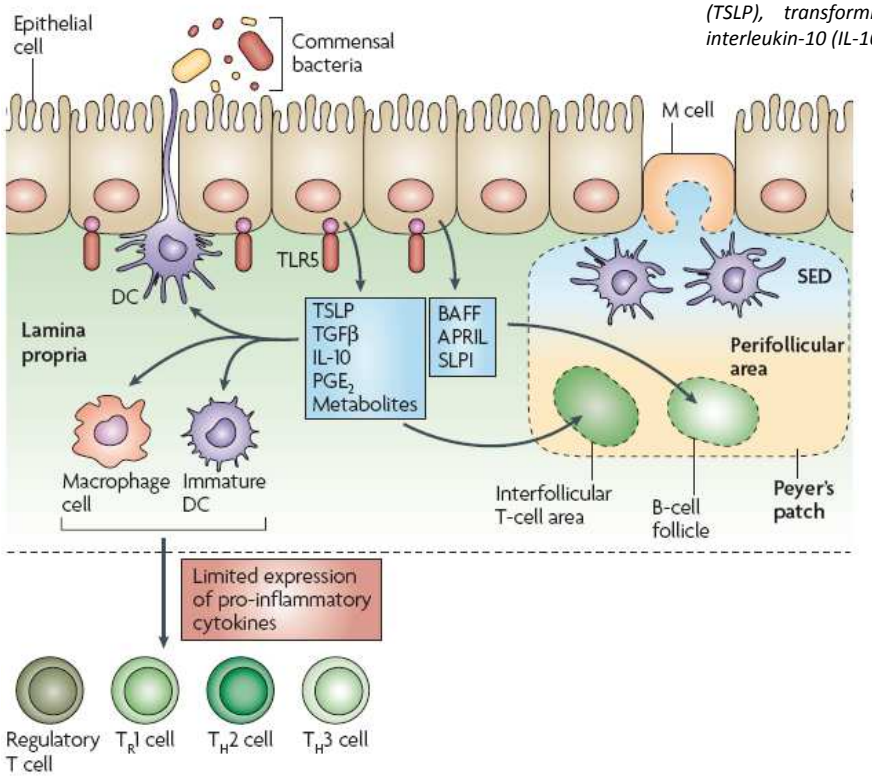
2. Invariant Natural Killer T Cells

iNKTs in the small intestine are present mainly in the lamina propia (Wingender and Kronenberg, 2008). They can be activated upon the presentation of lipid antigens by CD1d+ APCs (Chen and Ross, 2007). In addition, as we saw in chapter 4, sIECs act as non-professional APCs that express C1d (Chehade and Mayer, 2005). On the other hand, RARα agonists enhance CD1d expression *in vitro* in human monocytic cell lines through retinoic acid responsive elements. (Chen and Ross, 2007) This indicates towards a possible role of RA in lipid antigen presentation by sIECs to iNKTs in the gut.

3. Gamma-Delta T Cells

γδT cells are T cells that do not have the typical αβ chains in their T cell receptors, but γδ chains. They are considered as rapid lymphoid stress-surveillance cells that respond to tissue perturbation (Hayday 2009). These cells have been seen to be important for the induction of oral tolerance, but it is not quite clear yet how they influence this process (Ke et al. 1997). It is known, however that these cells can be activated by molecules produced by disrupted intestinal epithelial cells, such as retinoic acid early inducible-1 gene (RAE-1) in mice, and could be potentially regulated by RA metabolism. RAE-1 is involved in cell proliferation, and is also a ligand in NK and NKT cells. (Cédile et al. 2010)

Figure 13: Intestinal epithelial cells regulate immune-cell function. (Artis 2008)



Basal recognition of commensal bacteria by intestinal epithelial cells (IECs) may influence the secretion of cytokines, including thymic stromal lymphopoietin (TSLP), transforming growth factor-β (TGFβ) and interleukin-10 (IL-10), that can directly influence the cell expression of pro-inflammatory cytokines by dendritic (DC) and macrophage populations that resident in the lamina propia and Peyer's patches.

Signals derived from commensal bacteria may influence tissue-specific 'licensing' of accessory-cell functions resulting in the expansion and/or survival of T cells with regulatory capacities, including regulatory T cells, T regulatory type 1 (TR1) cells, T helper 2 (TH2) cells and TH3 cells. In addition to TSLP, TGFβ and IL-10, other IEC-derived factors, including APRIL (a proliferation-inducing ligand), B cell-activating factor (BAFF), secretory leukocyte peptidase inhibitor (SLPI), prostaglandin E2 (PGE2) and other metabolites have the capacity to directly regulate the functions of both antigen-presenting cells and lymphocytes in the intestinal microenvironment. TLR5, Toll-like receptor 5; SED, subepithelial dome.

B. sIECs and the GALT

Small intestinal epithelial cells provide the specific environment for the differentiation of certain specific behaviours of the innate immune cells in the gut (Figure 13). Mucosal dendritic cells in the gut are thought to be primed by intestinal epithelial cells through soluble factors to express the CD103 marker (Edele et al. 2009). These CD103+ DCs tend to regulate immune responses and induce other cells from the adaptive immune system to become tolerogenic FoxP3+ cells or to express with gut-homing proteins (Iliev et al. 2009, Edele et al. 2009, Molenaar et al. 2009 and Coombes et al. 2007).

Intestinal epithelial cells can also act as APCs and present antigen directly to T cells (Mowat 2003). They are the first cells to encounter invading pathogens and/or be stimulated by TLR ligands. They therefore produce cytokines and chemokines that modulate and potentially activate immune responses (Artis 2008).

"We must not cease from exploration and the end of all our exploring will be to arrive where we began and to know the place for the first time"
Thomas Stearns Eliot

6. Conclusions: Linking RALDH Expression in sIECs to Mucosal Innate Immune Regulation

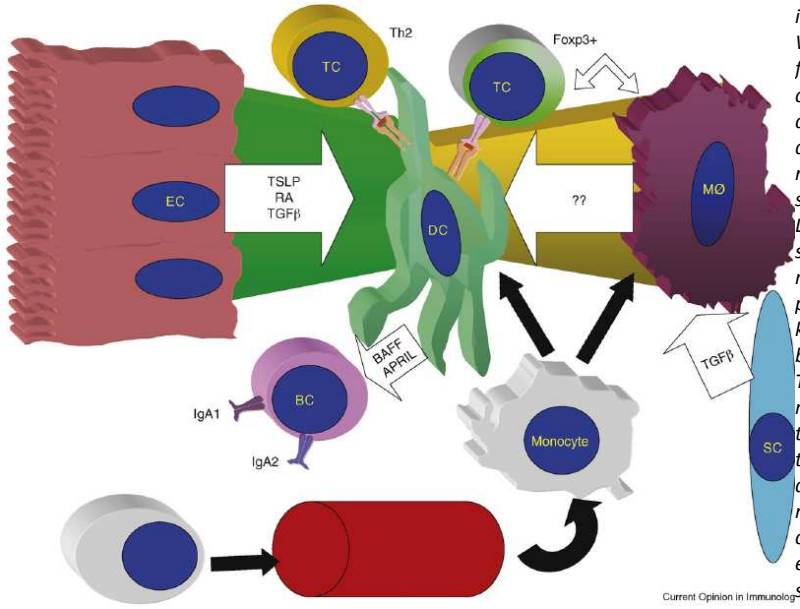


Figure 14: Epithelial cell–dendritic cell-macrophage interactions. Rescigno et al. 2009
What drives the development of the specialized functions of gut DCs? Human intestinal epithelial cells release TSLP, TGF-ß, and RA that drive the development of tolerogenic DCs able to induce Th2 and Foxp3+ Tregs. TSLP is also shown to favour the release of BAFF and APRIL by conditioned DCs and supports IgA class switching of B cells directly in the LP or the generation of protease-resistant IgA2 after sequential class switching from IgA1. Additionally, macrophages appear to tune the inflammatory potential of DCs, but the factors involved are not yet known. On the other hand, macrophages are shaped by stromal cell derived TGF-ß and induce Foxp3+ Tregs in a fashion similar to DCs. DCs and macrophages derive from circulating monocytes that could undergo 'mucosal' conditioning during their terminal differentiation into the tissue. In agreement, Tregs can steer the differentiation of monocytes into regulatory macrophages. Hence, the concerted action of immune cells, stromal cells, and epithelial cells is required to keep peace at intestinal surfaces.

The production of RA by sIECs has been shown to be fundamental for the induction of regulatory T cells in the intestinal mucosa. However it has been seen that this regulation is done through soluble factors that first regulate the production of RA by APCs in the gut (Iliev et al. 2009). RA produced by sIECs affects the levels of RALDH in APCs. This in turn affects a whole array of other cells under the influence of RA. T cells eventually differentiate into Th_2 cells or become regulatory T cells (Rescigno et al. 2009) (See Figure 14). It allows migration and phagocytosis by macrophages and neutrophils, it induces the migration of DCs, IgA production by B cells, and the expression of gut-homing molecules by T and B cells (Durianick et al. 2010) (See Figure 15). The ways in which RA affect all these processes is largely unknown although some are beginning to be elucidated.

What can be said is that all this is related to the production of RA by intestinal epithelial cells. RALDH being an essential enzyme for the production of RA is a key molecule that could influence the regulation of all these processes.

The distribution of RALDH along the tip-crypt axis is still disputed (Thomas et al. 2005) (Merlos-Suárez et al. 2011). To know exactly this distribution would help understand the immune processes that RALDH expression might influence. However, the profile along the

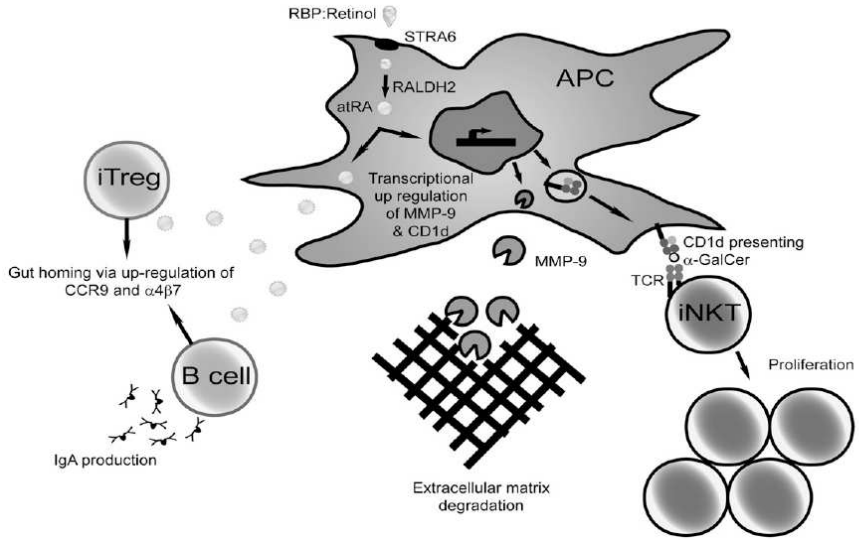


Figure 15: The metabolism and function of vitamin A in APC. Durianick et al. 2010 The APC expresses STRA6, which binds RBP and allows the APC to acquire retinol. The APC expresses RALDH 2 for the metabolism of retinol to atRA. Secreted atRA, synthesized by the APC, acts on CD4+ T lymphocytes to induce a regulatory T cell phenotype (iTreg) and stimulates isotype class switching to IgA by B lymphocytes. In addition, both B and T lymphocytes up-regulate CCR9 and α4β7 in response to atRA, leading to the homing to gut mucosa. Synthesized atRA up-regulates transcription of MMP-9 and CD1d by the APC. CD1d presents lipid antigens such as a-GalCer to invariant iNKT cells causing their proliferation. The secretion and activation of MMP-9 degrades gelatine, types I and IV collagen, and laminin in the extracellular matrix.

proximal-distal axis of the small intestine has been established (Alnouti and Klassen, 2008) (Molenaar 2010). Both genetic and environmental factors seem to affect this distribution: higher in the proximal part, and lower in the distal part.

A series of endogenous and dietary molecules, as well as several transcription factors that could affect the expression of RALDH in small intestinal epithelial cells, have been identified. They are mainly related to the activation of detoxifying enzymes and lipid metabolism. It would be interesting to see if these molecules have an effect on the regulation of innate immune signalling in the intestine.

Understanding the environmental regulation of RALDH enzymes could lead towards a better appreciation of how vitamin A metabolism and its consequences on the immune system could be affected by other components of the diet. This could play a vital role in dietary supplements in countries where the population have vitamin A deficient diets and increase its efficacy.

"The trouble with having an open mind, of course, is that people will insist on coming along and trying to put things in it."
Terry Prachett

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