Control of mucosal immune responses by vitamin A

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Table of contents

List of abbreviations

ADH	alcohol dehydrogenase
BM-DC	bone marrow derived dendritic cell
CM	chylomicron
CP	colonic patch
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
DC	dendritic cell
DSS	dextran sulphate sodium
E-lig	E-selectin ligand
GALT	gut associated lymphoid tissue
GM-CSF	granulocyte macrophage colonoy-stimulting factor
i.c.	intracutaneous
i.g.	intragastric
i.m.	intramuscular
IBD	inflammatory bowel disease
IFNy	interferon gamma
IgA	immunoglobulin type A
IL-2	interleukin-2
LRAT	lecithin:retinol acetyltransferase
LTbr	lymphotoxin beta receptor
LTi	lymphoid tissue inducer cell
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MHCII	major histocompatibility complex II
MLN	mesenteric lymph node
OVA	ovalbumin
P-lig	P-selectin ligand
PLN	peripheral lymph node
PP	Peyer's patch
PRR	pathogen recognition receptor
RA	retinoic acid
RALDH	retinaldehyde dehydrogenase
RAR	retinoic acid receptor
RARE	retinoic acid response element
RBP	retinol binding protein
RE	retinyl ester
RORyt	RAR-related orphan receptor
SIEC	small intestinal epithelial cell
SILT	solitary isolated lymphoid tissue
TGFb	transforming growth factor b
TLR	Toll-like receptor
VAC	vitamin A control
VAD	vitamin A deficient
VAH	vitamin A high

1

General Introduction

1

Index

The importance of vitamin A for the immune system has been known for a long time from epidemiological studies in developing countries where a large number of people, mainly children, suffer from vitamin A deficiency. Vitamin A deficiency compromises proper mucosal immune responses, causing diarrhea, infections and early childhood mortality [1-3]. The exact role for vitamin A in maintaining immune homeostasis was not known, but since the discovery in 2004 by Iwata *et al.* [4], that the vitamin A metabolite retinoic acid directly imprints T cells with gut-homing capacity, the role of vitamin A in the intestinal immune system has gained considerable attention from research groups.

The generic term "retinoids" covers hydrophobic, lipid-soluble and small sized molecules that include natural vitamin A (retinol) and all vitamin A metabolites, e.g. all-trans retinoic acid.

1. Vitamin A metabolism

1.1 Absorption, metabolism and storage

Vertebrate animals need vitamin A for several life processes such as embryogenesis, vision, reproduction, intestinal barrier function, immune function and normal cell proliferation and differentiation. The ability for de novo synthesis of vitamin A molecules is limited to plants and microorganisms [5,6]. Vertebrate animals, however, must obtain vitamin A from their diet. Vitamin A (retinol) is a fat soluble vitamin and is absorbed from the gastrointestinal tract. The major sources of retinol in human diet are provitamin A carotenoids which can be obtained from fruits and vegetables, or retinyl esters (REs) found in animal source food [7]. Food with the highest concentration of REs in western countries are livers, fortified foods, milk, eggs or margarines. The highest concentration of provitamin A carotenoids can be found in carrots, pumpkin, spinach and kale [8]. In the intestinal lumen, carotenoids and REs are metabolized by endogenous enzymes secreted into the lumen to generate free retinol prior to absorption by enterocytes (subset of intestinal epithelial cells, Figure 1) [9-11]. Within enterocytes, retinol will be bound to cellular retinol binding protein 2 (CRBP2) [12]. The CRBP2-retinol complex serves as a substrate for the esterification process of retinol to REs by lecithin:retinol acyltransferase (LRAT) [13,14]. All formed REs are incorporated into chylomicrons or intestinal lipoproteins and then released into the lymphatic system [7,15,16] and the portal circulation [17] for transport to liver hepatocytes and stellate cells (Figure 1). The liver is the primary site of vitamin A storage, where vitamin A will be stored mainly in the form of REs in large cytoplasmic lipid droplets [18-22]. Liver stellate cells account for 80% of total body vitamin

A storage, while the remainder is stored in liver hepatocytes [18,23]. The liver regulates the availability of serum retinol to meet tissue requirements. Before REs are mobilized from their storage, REs are hydrolyzed into free retinol that will bind to serum retinol binding proteins (RBPs) for secretion from the liver, transport through the circulation and delivery to target cells (Figure 1) [24]. Since retinol is relatively hydrophobic, it requires protein binding to be effectively transported. Also, binding to RBPs prevents retinol from chemical and enzymatic degradation. In vitamin A sufficient animals, circulating levels of RBP-retinol are maintained at a constant level. These levels generally do not decline, except when hepatic stores of vitamin A are depleted. The levels also decrease in the setting of serious infections, however the regulation of serum retinol levels by the liver is not completely understood.

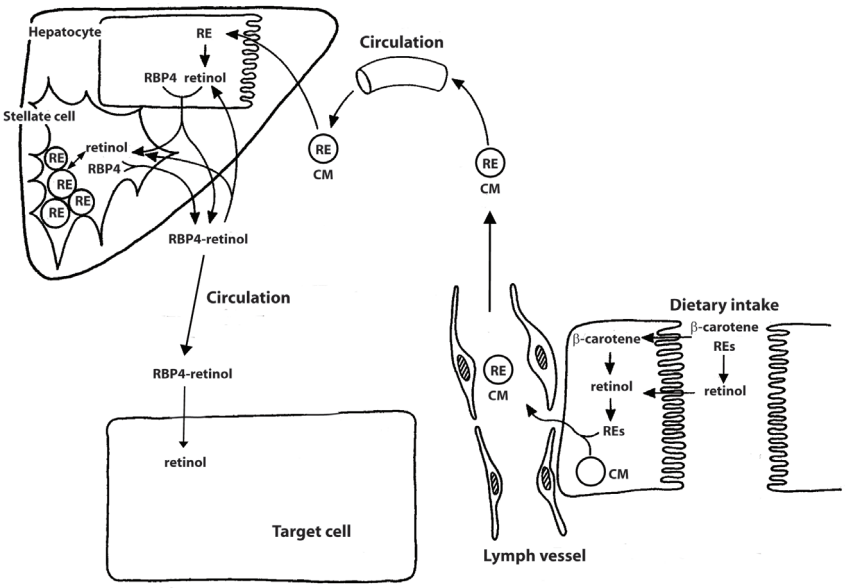

Figure 1. Major pathway for vitamin A absorption and transport (adapted from [221]).

Dietary retinyl esters are hydrolyzed to retinol in the intestinal lumen. Retinol and carotenoids are absorbed by enterocytes and converted into retinyl esters to be packaged into chylomicrons. Chylomicrons are released into the lymph and subsequently reach the circulation. Chylomicrons are cleared from the blood by the liver, where retinyl esters are hydrolyzed to retinol in hepatocytes. Here retinol will be bound to retinol binding proteins for transfer to stellate cells where retinol can be stored in lipid droplets in the form of retinyl esters. Alternatively, retinol can be released into the circulation bound to retinol binding proteins for delivery of retinol to target cells. RE, retinyl ester; CM, chylomicron; RBP, retinol binding protein.

1.2 Retinol delivery to target cells

Target cells expressing a RBP receptor on the plasma membrane can recognize retinol-RBP complexes and mediate the cellular uptake of retinol from its complex. STRA6 is now described as a cell surface receptor for retinol-RBP complexes. STRA6 is a member of a large group of "stimulated by retinoic acid" (STRA) genes that encode transmembrane proteins and it specifically binds to retinol-RBP complexes to mediate retinol uptake from its complex into the cell (Figure 2) [25-27]. RNAi knockdown of STRA6 in WiDr cells greatly diminished retinol uptake [25]. Consistent with the essential roles of vitamin A in human development, mutations in human STRA6 caused severe birth defects, reduced RBP binding and largely abolished vitamin A uptake activity [26,28,29]. STRA6 is widely expressed in the murine embryo and in the adult, yet STRA6 is undetectable (or is expressed at low levels) in some tissues that are highly responsive to retinoids like the skin or liver [29]. This suggests the existence of other RBP receptors that have yet to be discovered.

1.3 Cellular retinoid binding proteins

Various cellular retinoid binding proteins exist to solubilize and stabilize their hydrophobic and labile ligands in the aqueous milieu within cells. These binding proteins show high specificity and affinity for their specific retinoid ligands. In addition, cellular retinoid binding proteins have specific functions in the regulation of retinoid transport, metabolism and activity of retinoids [30] (Figure 2). The cellular retinoid binding proteins include cellular retinol binding proteins (CRBPs) and cellular retinoic acid binding proteins (CRABPs) and belong to a family of cytosolic proteins binding small hydrophobic ligands [31,32]. In general, the cellular concentrations of CRBPs and CRABPs exceed the concentrations of their ligands. CRBPs are known to exist in 4 isoforms of which CRBP1 and CRBP2 are the most well-known retinol binding proteins. CRBP2 expression is almost exclusively restricted to adult intestines and is involved in absorption of retinol from the intestinal lumen [33-36]. CRBP1 is expressed in multiple extra-intestinal tissues and is involved in uptake of retinol from the circulation [30,37]. In addition, CRBPs are proposed to facilitate the metabolism of retinol, since enzymes like LRAT or alcohol dehydrogenases (ADHs) recognize the CRBP-retinol complex very efficiently, while the affinity of these enzymes for free retinol is much lower. CRBPs promote access of retinoids to some enzymes, while metabolism or catabolism by other enzymes is prevented.

A second family of retinoid binding proteins is formed by the cytoplasmic cellular retinoic acid binding proteins (CRABPs). The general function of CRABPs is to allow access to enzymes for generation of retinoic acid and modulate the concentration of retinoic acid available to nuclear retinoid receptors [38]. Two isoforms, CRABP1 and CRABP2, exist that are highly similar, displaying about 74% sequence identity,

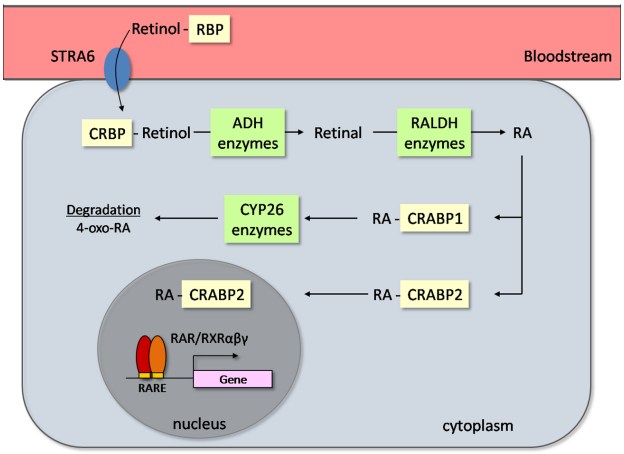

Figure 2. Intracellular vitamin A metabolic pathway.

Retinol is transported through the bloodstream by RBP. Cells expressing retinol-RBP binding receptors like STRA6 specifically bind RBP and mediate retinol uptake from the retinol-RBP complex into the cell. Within the cytoplasm, retinol forms a complex with CRBP and is subjected to conversion by ADH enzymes for metabolism of retinal. RALDH enzymes subsequently produce RA from the CRBP-retinal complex. RA is bound to either CRABP1 or CRABP2. CRABP1 functions by targeting RA to cytochrome P-450 CYP26 enzymes that degrade RA to inactive metabolites like 4-oxo-RA. CRABP2 undergoes rapid nuclear localization upon binding RA and delivers RA to its retinoic acid receptors (RARs and RXRs). These receptors bind to short DNA sequences in the vicinity of target genes known as RAREs. RA receptors act as transcription factors that will regulate the transcription of many target genes upon binding of RA.

ADH, alcohol dehydrogenase; CRABP, cellular retinoic acid binding protein; CRBP, cellular retinol binding protein; RA, retinoic acid; RARE, retinoic acid response element; RALDH, retinaldehyde dehydrogenase; RBP, retinol binding protein.

and are highly conserved between species. Yet, these isoforms display different patterns of expression across cells and have different specific functions. CRABP1 is almost ubiquitously expressed, while CRABP2 is more specific to certain cell types. CRABP1 is predominantly present in the cytoplasm and almost excluded from the nucleus. CRABP1 serves to target retinoic acid to cytochrome P-450 CYP26 enzymes that degrade retinoic acid to inactive metabolites (Figure 2) [39,40]. Increased expression of CRABP1 leads to enhanced retinoic acid degradation rates [41]. CRABP1 binds retinoic acid with a 5-fold higher affinity than CRABP2 [38,42] and thus acts to decrease cellular retinoic acid concentrations and to diminish cellular responses to retinoic acid. CRABP2, a predominantly cytosolic protein, acts to deliver retinoic acid to the nucleus [43]. CRABP2 undergoes rapid nuclear localization upon retinoic acid binding and interacts with retinoic acid receptors (RARs) in a ligand-dependent fashion to deliver retinoic acid to its receptor for regulation of gene transcription (Figure 2) [44]. Overexpression of CRABP2, but not CRABP1, results in a marked stimulation of transcription of RAR-driven reporter genes [44-46]. Interestingly, cells that lack CRABP2 are not responsive to retinoic

acid [47,48], further corroborating that CRABP2, and not CRABP1, is responsible for delivery of retinoic acid to the nucleus. In conclusion, expression levels of CRBPs regulate retinol uptake and metabolism, while CRABPs control cellular retinoic acid concentrations and cellular responses to retinoic acid. Therefore, the expression levels of these binding proteins within cells are critically important for retinoid dependent signalling.

1.4 Retinoic acid metabolism and catabolism

In order to fulfill its function vitamin A (retinol) must be metabolized to retinoic acid, the active form that can regulate gene expression by serving as a ligand for nuclear retinoic acid receptors. Retinol itself is inactive and does not bind to nuclear receptors. After cellular uptake, retinol, complexed with CRBP, is subjected to enzymatic metabolism. The major pathway of retinoic acid synthesis depends on two steps (Figure 2). In the first step, retinol is reversibly oxidized by alcohol dehydrogenases (ADH) to form retinaldehyde (retinal). Within the vertebrate ADH family, ADH1 and ADH4 have been demonstrated to metabolize retinol to retinal in both human and mouse [49-51]. In the second step, retinal is irreversibly metabolized to retinoic acid by retinaldehyde dehydrogenases (RALDH) [52-54]. The vertebrate RALDH family consists of 16 distinct enzymes with RALDH1, RALDH2 and RALDH3 representing the three cytoplasmic enzymes producing retinoic acid from CRBP-retinal. RALDH1 through 3 are highly conserved between man and mouse. RALDH1 was the first enzyme found to oxidize retinal to retinoic acid in man, originally called ALDH1, as well as in mouse, originally called Ahd-2 [55,56]. RALDH1 expression is found in a subset of retinoid-dependent embryonic tissues, as well as in many adult epithelia [57]. RALDH2 shares 72% sequence homology with RALDH1. The catalytic efficiency of RALDH2 for retinal oxidation is about 15-fold higher than that of RALDH1 [58,59]. RALDH2 expression occurs in many retinoid-dependent embryonic tissues and in adult reproductive organs [57]. RALDH3 was identified much later to be conserved between mouse and man and being able to metabolize retinoic acid from retinal [52]. All-*trans* retinoic acid is the most potent biologically active vitamin A metabolite produced by RALDH enzymes and can prevent and rescue the main defects caused by vitamin A deficiency in adult animals [60]. Other isoforms of retinoic acid are amongst others 13-***cis*** retinoic acid, specific for the eye, and 9-***cis*** retinoic acid. The consideration of 9-***cis*** retinoic acid as a natural bioactive metabolite remains controversial since 9-***cis*** retinoic acid has not been consistently detected in mammalian cells.
Retinoic acid has a short half life *in vivo* and *in vitro* of about 6-7 hours [61,62]. Cytochrome P-450 enzymes are involved in the degradation of endogenous retinoic acid into inactive metabolites, like 4-oxo retinoic acid, to regulate cellular retinoic acid levels and to protect cells from excess retinoic acid [39,40,63,64].

1.5 Transcriptional gene regulation by retinoic acid

Once formed, the vitamin A metabolite retinoic acid serves as a ligand for nuclear retinoic acid receptors (RARs and RXRs) for regulation of target gene expression. Nuclear receptors are ligand-dependent transcription factors and regulate gene expression of specific subsets of genes by binding to short DNA sequences in the vicinity of target genes known as retinoic acid response elements (RAREs) [65]. Most identified RAREs have been found to be direct or inverted repeats of consensus sequence (A)GGTCA spaced by 1, 2 or 5 basepairs [66-69]. Three genes encoding highly related RARs have been identified in mouse and human, RARα [70,71], RARβ [72] and RARγ [73,74]. The RAR genes are mapped on different chromosomes [74]. All-*trans* retinoic acid as well as synthetic retinoic acid analogues are high-affinity ligands for these three RARs. Other retinoic acid isomers, like 9-***cis*** retinoic acid, bind RARs with low affinity and specifically bind (with higher affinity) to RXRs. Three distinct RXR genes (RXRα, RXRβ, and RXRγ) have been found in mouse and man [75-77]. RARs and RXRs belong to two different groups of the nuclear receptor family, but both are involved in retinoid signalling. RARs have been studied intensively, but multiple questions about the actual biological role of RXRs have still to be answered.

At least one of the RARs has been found in every cell type examined, and sometimes two or three of the RARs are expressed in a given cell type. For high affinity binding to RARE sequences, RARs heterodimerize with RXRs, while homodimers of RARs can only bind RARE sequences at high protein concentration [78-80]. In the absence of RAR ligand, the RAR-RXR dimers recruit corepressor proteins (like NCoR,

Figure 3. Schematic representation of retinoic acid receptor proteins (adapted from [79]).

Retinoic acid receptors (RARs and RXRs) have a well-defined domain organization and structure composed of six conserved regions, A to F. Regions C and E are the most conserved and important domains for transcriptional regulation of target genes. The C region contains the central DNA-binding domain (DBD) that recognizes retinoic acid response elements (RAREs) composed of (A)GGTCA repeats in the DNA of target genes. The E region contains the ligand-binding domain (LBD) for retinoic acid (RA). The ligand-binding pocket in the LBD contains hydrophobic residues and the shape of the ligand binding pocket matches the volume of the RA ligand. This maximizes hydrophobic contacts and contributes to the selectivity of ligand binding [222, 223]. The E region also contains a heterodimerization surface for pairing of RARs and RXRs and sites that interact with coregulators. The N-terminal domain (NTD) corresponds to regions A and B that interact with specific coregulators. The D region is considered to serve as a hinge between the DBD and the LBD, allowing rotation of the DBD. It also harbors nuclear localization signals. The F region, which is absent in RXRs, has multiple phosphorylation sites that might modulate RAR function, but the exact functions of this region remain poorly understood. NTD, N-terminal domain; DBD, DNA-binding domain; LBD, ligand-binding domain.

SMRT, HDACs) that lead to an inactive condensed chromatin structure, preventing transcription. When retinoic acid binds to RAR-RXR dimers, corepressors are released and coactivator complexes (amongst others histone acetyltransferases or methyl transferases) are recruited to activate gene transcription [81] (Figure 3). RARs have a well-defined domain organization and structure, consisting of a central DNA binding domain linked to a C-terminal ligand-binding domain (LBD) and an N-terminal domain, which interacts with specific coregulators [82] (Figure 3). Degradation of RARs by the ubiquitin-proteasome controls the magnitude and duration of the retinoid response [83-85].

A large number of the retinoic acid-responsive genes encodes proteins that participate in the metabolic pathway of vitamin A. For instance, all three RAR genes contain a RARE site in their promoters [67,86-88]. Retinoic acid also regulates expression levels of CRBP1 and CRBP2 [89,90], LRAT [91,92] as well as CRABP2 [68,93]. We and others have demonstrated that the RALDH2 gene is also responsive to retinoic acid [58,94,95] (see chapter 4). Furthermore, retinoic acid induces its own catabolism by inducing CYP26 transcription [96]. In this way cellular retinol uptake is maintained by inducing CRBP, but retinoic acid concentrations are limited by accelerating its catabolism and preventing cellular retinoic acid toxicity. retinoic acid itself therefore functions as a regulator of its own synthesis and catabolism creating an autoregulatory loop.

Table 1. Players in retinoic acid-dependent signalling

		Functions
Ligand	*All-trans retinoic acid*	*Active vitamin A metabolite*
		Receptor specificity
		Cell-specific synthesis
Binding proteins	*RBP*	*Transport, Metabolism, Sequestration*
	CRBP1 and CRBP2	
	CRABP1 and CRABP2	
Nuclear receptors	*RARα, RARβ, RARγ*	*Transcription factors*
	RXRα, RXRβ, RXRγ	*DNA-binding*
		Ligand, cell and promoter specific activity
Receptor dimers	*RAR/RXR*	*Combinatorial diversity*
Response elements	*RARE and RXRE*	*Regulation gene transcription*
	Direct or inverted	*Activation or repression*
	repeats of (A)GGTCA	

1

2. Global prevalence and intervention of vitamin A deficiency

The importance of vitamin A can clearly be inferred from situations of vitamin A deficiency where epithelial integrity, growth and immunity are impaired. Vitamin A deficiency weakens the host defense against infection, causing increases in the incidence and severity of infections and ultimately elevates the risk of mortality in early childhood [97,98]. Vitamin A deficiency contributes to the deaths of over a million children each year, especially from measles, diarrhea and malaria [99,100]. The most recent estimates from the World Health Organization report that in developing countries about 190 million preschool children (~32%) and nearly 20 million pregnant mothers (~10%) are vitamin A deficient (Figure 4) [3]. Improving the vitamin A status of young children in developing countries has been shown to reduce child death rates by 20–50%, which suggests that a substantial portion of their mortality is attributable to vitamin A deficiency [101].

Vitamin A deficiency is defined when liver stores of vitamin A fall below 20 μg/g (0.07 μmol/g) and serum retinol levels below 20 μg/dL (0.70 μmol/L) [102,103]. Under normal conditions, serum retinol levels are 1-3 μmol/L. As vitamin A status declines physiological functions become impaired. Initially, the integrity of epithelial barriers (surface linings of the gastrointestinal, respiratory, excretory and reproductive systems) is disturbed and the immune system becomes compromised, followed by impairment of the visual system. Consequently, there is progression to total blindness, increased severity of infections and an increased risk of death, especially among children. Other clinical signs of vitamin A deficiency include growth retardation and anemia [100,104].

The underlying cause of vitamin A deficiency is inadequate consumption of vitamin A rich foods which is related to poverty. In general, young children in industrialized countries receive most of their vitamin A from animal source food, whereas provitamin A in fruits and vegetables is the primary source of dietary vitamin A for children in underdeveloped countries [101,105]. Due to poverty, people turn to low priced food of lower quality and variety, which limits consumption of vitamin A rich foods [3]. This becomes evident when physiological needs are greatest, namely during early childhood, pregnancy, and lactation. Vitamin A deficiency in children is initially caused by the fact that their mothers are deficient and produce breast milk with very low concentrations of vitamin A [101]. Generally, all newborn children are physiologically vitamin A “depleted” at birth and need to build up adequate vitamin A stores in their livers. Breast fed babies of vitamin A-deficient women therefore remain depleted. Furthermore, if these children receive food after breast feeding that is also low in vitamin A, the child’s risk for developing vitamin A deficiency further increases when breast feeding stops. A third contributing factor is that they spend a substantial part of childhood being sick, suffering from diarrhea and infections. These illnesses involve loss of appetite and malabsorption of nutrients, which further deteriorate

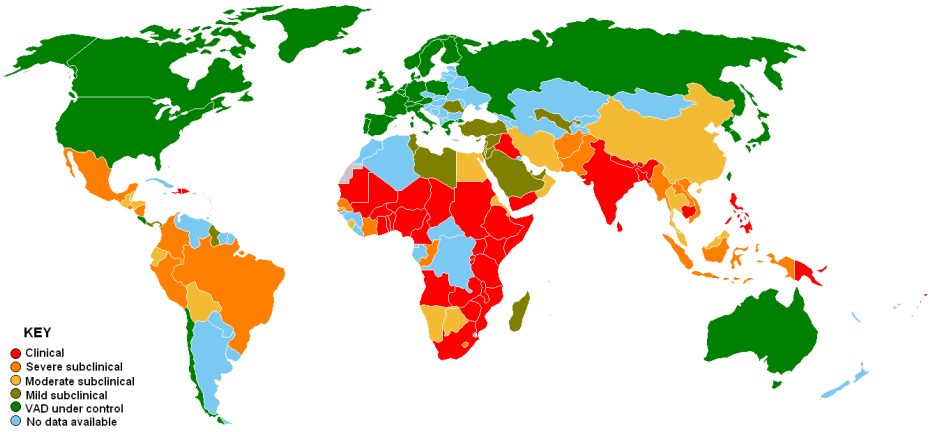

Figure 4. Global prevalence of vitamin A deficiency

This map displays the prevalence of vitamin A deficiency (VAD) worldwide (from WHO Global database on vitamin A deficiency). Mainly in underdeveloped countries, vitamin A deficiency poses a major public health problem. Vitamin A deficiency is categorized in clinical (red) to severe (orange), moderate (yellow,) and mild (light green) subclinical symptoms in individuals. Vitamin A deficiency is under control in western countries (green).

their vitamin A status [101].

Young children in developing countries cannot build up adequate liver vitamin A stores in the liver from plant diets alone, since dietary intake is too low. The efficiency with which plant-derived provitamin A can be converted into vitamin A is much lower than that for preformed vitamin A in animal source foods [105]. To overcome or prevent vitamin A deficiency, consuming modest amounts of animal or fortified food sources of preformed vitamin A or vitamin A supplementation is required. The first randomized trial of vitamin A supplementation was started in 1986 in Indonesia, after which it was repeated in other populations in the late 80s and early 90s [2]. These trials demonstrated a clinically and statistically significant reduction (~29-54%) in mortality among children from 6 months to 5 years of age [2]. By 1992, consensus was reached that vitamin A deficiency increased overall mortality and improving vitamin A status would reduce this. Vitamin A supplementation is a very effective approach to increase vitamin A intake and is being developed to correct vitamin A deficiency worldwide. The World Health Organization (WHO) recommends children to receive a defined dose of vitamin A once every four to six months. The great majority (78%) of countries where vitamin A deficiency is known to be a major public health problem have adopted this policy and vitamin A supplementation of children has been successfully integrated into routine maternal child health visits, or is often linked to community-based nutrition improvement programs like National Immunization Days or Vitamin A week [2]. Other effective means to control and prevent widespread vitamin A deficiency are fortification of foods with vitamin A and supplementing pregnant

mothers with vitamin A resulting in improved health of the mothers and increased vitamin A levels in breast milk. This subsequently will reduce childhood mortality [100,106-108].

In conclusion, the prevention of vitamin A deficiency remains a global public health priority. With the continuation of present supplementation programs, clinical vitamin A deficiency may be eliminated in many parts of the world in the coming years. Nevertheless, many children will remain affected by sub-clinical vitamin A deficiency. Therefore, it will be necessary to pursue intervention programs to allow long-term control of vitamin A deficiency and improve health and nutritional status in developing countries. Although vitamin A supplementation has taken its place as a major health intervention, it is still not precisely known how it improves health and the immune system and increases resistance to infection. Research has now focused on the protective mechanisms of vitamin A to provide more insights into the role of vitamin A in immune function.

Box 1. Hypervitaminosis

Worldwide the incidence of vitamin A excess, or hypervitaminosis A, is a very minor problem compared with the incidence of vitamin A deficiency. A few hundred cases of hypervitaminosis A occur annually whereas an estimated 1 million people develop vitamin A deficiency each year. However, vitamin A toxicity may be a growing concern because intake from preformed sources of vitamin A often exceeds the recommended dietary allowances (RDA) [109]. Assessing vitamin A status in persons with vitamin A toxicity is difficult since serum retinol levels are tightly regulated and are therefore inadequate indicators of liver vitamin A stores [109]. Hypervitaminosis A may result from excessive consumption of animal source food or overconsumption of vitamin A supplements and can be divided into two categories: acute, resulting from ingestion of a very high dose over a short period of time, and chronic, resulting from continued ingestion of high doses for months or even years.

Typical symptoms of acute hypervitaminosis A include bulging fontanels in infants, headache due to increased intracranial pressure in adults, nausea, vomiting, fever, vertigo, hemorrhages, joint pains, and visual disorientation [110-112]. The symptoms are generally transient and do not lead to permanent adverse effects.

Chronic hypervitaminosis A is more common than acute hypervitaminosis A. Its symptoms are highly variable but anorexia, skin problems, loss of hair, increased intracranial pressure, and hepatomegaly are among the most common symptoms [110-112]. Osteoporosis and hip fractures have now also been associated with chronic overcomsumption of vitamin A of only twice the RDA [113-115]. This involves hypercalcemia, increased bone resorption and decreased bone formation [116-118]. Moreover, excessive intake of vitamin A by pregnant women can cause abnormal morphological development and birth defects in the newborn child [111,119].

3. The mucosal immune system

The intestinal immune system has to be non-responsive and be able to induce tolerance towards harmless food antigens and the commensal microflora, while robust immune responses are needed towards harmful pathogens. Maintenance of this critical balance is attributed to mucosal dendritic cells (DCs) residing in gut-associated lymphoid tissues, like Peyers' Patches (PPs) and mesenteric lymph nodes (MLNs), and in the subepithelial lamina propria of the intestines. Dysregulation of this balance results in uncontrolled inflammatory disorders such as inflammatory bowel diseases in humans. Many studies have shown the beneficial effect of vitamin A on the mucosal immune system and research now concentrates on the mechanisms by which vitamin A and its metabolite retinoic acid maintain intestinal homeostasis.

3.1 Lymphocyte subsets and retinoic acid

Th1 versus Th2 cells

Naïve $CD4^+$ T cells, produced in the thymus, can become Th1, Th2 or Th17 cells, which act as effector/memory cells to stimulate the immune system to clear pathogens and tumor cells, or can become regulatory T cells which function to suppress the immune system. The subset of T cells termed Th1 cells, which produce interleukin-2 (IL-2), interferon-γ (IFNγ) and lymphotoxin, are in particular important for immunity against intracellular pathogens, such as *Leishmania Major*. By their production of IFNγ, they deliver assistance to macrophages for enhanced pathogen clearance. In contrast, Th2 cells produce IL-4, IL-5, IL-10 and IL-13, which contributes to the direct enhancement of antibody-mediated immunity against extracellular pathogens. Dietary vitamin A has been shown to affect Th1 and Th2 development as vitamin A deficiency causes IFNγ overproduction and shifts the immune response toward a Th1-type response and impairs the development of a Th2-type response [120,121]. On the other hand, high-level dietary vitamin A enhances Th2 cytokine and decreases Th1 cytokine production [122]. Moreover, it was shown that the vitamin A metabolite retinoic acid directly skews T cells upon their activation towards the Th2-type profile, while inhibiting Th1 cytokine production [123,124]. Via RAR signalling, retinoic acid can directly suppress IFNγ production and enhance IL-4 production in stimulated T cells *in vitro*. Specific blockade of RAR, and not RXR, further confirmed the crucial involvement of RAR signalling in retinoic acid-mediated Th2 skewing [124]. Therefore, the balance of Th1 and Th2 responses is highly dependent on vitamin A status.

FoxP3 expressing regulatory T cells

In addition to Th1 and Th2 cells, regulatory T cell (Treg cells), identified by FoxP3 expression, are an essential component of the immune system. Treg cells function to induce tolerance to harmless food antigens or commensal bacteria and prevent escalation of immune responses. FoxP3$^+$ Treg cells are made in the thymus from progenitors. In addition, immunization through the oral route results in the induction of Treg cells from naïve CD4$^+$ T cells in peripheral lymphoid organs [125-128].

The cytokine milieu is very important in the induction of FoxP3$^+$ Treg cells. TGFβ and IL-2 are well known for their role in the induction and maintenance of FoxP3$^+$ Treg cells [129-131]. More recently, retinoic acid was identified as a cofactor that controls peripheral induction of FoxP3$^+$ Treg cells in mice [123,132-138]. Retinoic acid, in combination with TGFβ, is very effective in inducing FoxP3 expression in murine CD4$^+$ as well as murine CD8$^+$ T cells, while this induction could be blocked with RAR antagonists or neutralizing anti-TGFβ antibodies [135-138]. In contrast to the induction of Treg cells in mice, TGFβ is dispensable for human Treg cell conversion. Retinoic acid alone was shown to be sufficient to convert human naïve T cells into FoxP3$^+$ Treg cells [135].

The mechanism by which retinoic acid regulates human naïve T cells to convert to Treg cells occurs at multiple levels. By binding to the RARE site in the promoter region of Foxp3, retinoic acid induces histone acetylation in the human FoxP3 promoter [135,139], which is thought to open the promoter for enhanced transcription of the FoxP3 gene. In addition, retinoic acid enhances FoxP3 induction indirectly by relieving inhibition from CD4$^+$ CD44$^+$ effector/memory T cells [134], through reducing the production of inflammatory cytokines [134]. Further proof for the role of retinoic acid in the generation of FoxP3$^+$ Treg cells in mice has come from studies in which either retinoic acid or RAR antagonists were administered [135,136]. While administration of retinoic acid during antigenic stimulation resulted in an increase in Treg conversion [135,136], blockade of RAR signalling decreased the generation of FoxP3$^+$ Treg cells [136].

Th17 cells

More recently, a subset of inflammatory T cells named Th17 has been identified [140-142]. Th17 cells secrete IL-17, IL-21, and IL-22 [143-145] and have an important role in protecting the host from bacterial and fungal infections, particularly at mucosal surfaces. IL-23, IL-6 and TGFβ promote the differentiation of naïve T cells into Th17 cells [146-149] and are under the control of the transcription factor RORγt [150]. Since both Th17 and Treg cells are dependent on TGF-β as an inductive cytokine controlling their development, the levels of IL-6 become important. It has been shown that retinoic acid can directly counteract the IL-6 effect in a dose-dependent manner and that it reduces the expression of RORγt in T cells activated under Th17 culture conditions [136].

Whether inflammatory Th17 cells or suppressive FoxP3$^+$ T cells are generated largely depends on the local cytokine milieu formed by IL-6, TGFβ and retinoic acid (Figure 5). Under physiological conditions, the retinoic acid-driven, TGFβ-dependent Treg cell conversion might overrule the IL-6–driven TGFβ-dependent Th17 cell differentiation [136], while appropriate concentrations of TGF-β and IL-6, IL-21, and IL-23 might skew T cells towards Th17 development [146,151,152]. It is now well established that retinoic acid is very important for the generation of these T cell subsets, either by promoting or blocking the differentiation of naïve T cells into Th1, Th2, Th17 or Treg cells.

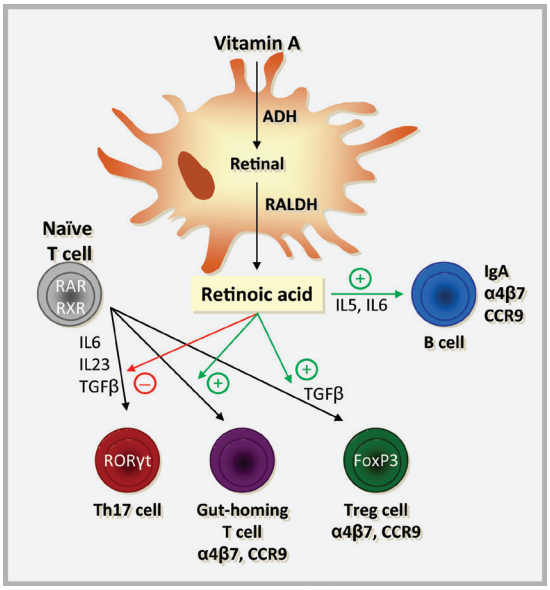

Figure 5. Retinoic acid synthesis by dendritic cells and its effects on lymphocytes.

Through the expression of ADH and RALDH enzymes, mucosal dendritic cells can produce retinoic acid from vitamin A. Retinoic acid acts on T cells and B cells by inducing the mucosal homing receptors α4β7 and CCR9. Moreover, retinoic acid in the presence of TGFβ promotes the conversion of naïve T cells into Foxp3$^+$ regulatory T cells and inhibits the differentiation of Th17 cells. In addition, retinoic acid synergizes with IL-6 and IL-5 and promotes class switching to IgA in B cells.

B lymphocytes

Retinoic acid is also physiologically important for immunoglobulin secretion by B cells. IgA is the most abundant immunoglobulin isotype produced in the body and serves to protect the host against the vast array of microbes constantly present in the intestinal lumen. Mice with defective or impaired IgA production are more susceptible for intestinal pathogens [153].

PPs and MLNs are the main secondary lymphoid tissues where naïve B cells will differentiate into IgA secreting plasma cells. Subsequently, these IgA producing B cells will migrate from MLNs and PPs to the lamina propria of the intestine where IgA is secreted into the lumen as part of the antimicrobial defense [154,155]. It has been shown that DCs from PPs, but not from spleen, promoted immunoglobulin class switching to the IgA isotype in activated B cells [156]. Furthermore, in the presence of retinoic acid, B cells displayed increased class switching to the IgA isotype [157] and IgA class switching induced by PP-DCs, MLN-DCs or lamina

propria DCs was diminished in the presence of RAR inhibitors [158-160]. Consistently, animals depleted of vitamin A show decreased IgA secretion and decreased mucosal antigen-specific IgA responses [159,161,162], while studies with high dietary vitamin A showed that IgA responses and IL-10 production were significantly enhanced [122]. Thus, retinoic acid has a direct IgA promoting effect on B cells which seems to be disturbed during vitamin A deficiency.

3.2 Immunomodulatory role of retinoic acid

Tissue-specific lymphocyte migration

It is well recognized that DCs play a central role in the priming and differentiation of naïve T cells into Th1, Th2, Th17 or regulatory T cells. Also, DCs can imprint trafficking programs in T cells that target their migration to specific peripheral tissues by the induction of specific combinations of adhesion molecules and chemokine receptors. Within the periphery, DCs acquire antigens from their surroundings, process it, and display the processed antigen on their surfaces using major histocompatibility class I or II molecules. When DCs arrive at the gut-draining MLN, they expose naive T cells to antigen and initiate T cell activation and proliferation, either inducing effector/memory T cells or tolerogenic regulatory T cells depending on the nature of the antigen. Upon activation by DCs, T cells are induced to express adhesion molecules and chemokine receptors to ensure that activated T cells will preferentially migrate to the site where DCs initially encountered the antigen [163-167].

The induction of tissue-specific homing mlecules upon lymphocyte activation within the draining lymph nodes enhances the efficiency by which effector lymphocytes will arrive at the site where their action is needed. Studies have shown that DCs from gut-associated lymphoid tissue (GALT), like MLN-DCs, PP-DCs or lamina propria DCs, have a selective ability to induce homing receptors on activated T cells specific for migration to the intestines, i.e. integrin $\alpha_4\beta_7$ and chemokine receptor CCR9 [166,168-172]. Homing of IgA secreting plasma cells to the intestinal lamina propria is also mediated by $\alpha_4\beta_7$ and CCR9 molecules [154,173,174].

The ligand for $\alpha_4\beta_7$, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), is expressed on endothelial cells in the lamina propria of the colon and small intestine [175-177], while CCL25, the ligand for CCR9, is produced by epithelial cells of the small intestine [178-183]. DCs in skin-draining lymph nodes or splenic DCs are unable to imprint gut homing molecules but instead induce skin-homing molecules on responding T cells. This imprinting results in the expression of the skin-homing molecules E- and P-selectin ligands as well as the chemokine receptors CCR4 and CCR10 on activated T cells [163,165,166,184-186]. Expression of these two chemokine receptors enables T cells to respond to CCL17

and CCL27 expressed by keratinocytes in the skin [187]. By expression of tissue-specific homing molecules, migration of effector/memory T cells and B cells to peripheral tissues is ensured.

Retinoic acid-dependent imprinting of gut-homing molecules

In 2004, it was discovered by Iwata et al. that the vitamin A metabolite retinoic acid directly affects gut-homing molecule expression on T cells. Gut-homing molecules $\alpha_4\beta_7$ and CCR9 could easily be induced on both $CD4^+$ and $CD8^+$ T cells in vitro when they were activated in the presence of retinoic acid [4]. Upon activation in the absence of retinoic acid, T cells expressed skin-specific homing molecules E- and P-selectin ligands, which were downregulated when retinoic acid was present [4]. In addition, the induction of $\alpha_4\beta_7$ and CCR9 expression by activated B cells also requires retinoic acid [159]. Experiments with transgenic T cells from luciferase reporter mice, in which luciferase activity is under the control of a promoter with RARE sites, furthermore showed that the induction of CCR9 required signalling of retinoic acid through its nuclear receptors and CCR9 induction was indeed blocked by the presence of a pan-RAR antagonist [188].
Further proof for the importance of vitamin A in the imprinting of lymphocytes with gut tropism came from studies with vitamin A deficient mice, in which secondary lymphoid organs as well as the intestinal lamina propria were depleted of $\alpha_4\beta_7^+$ $CD4^+$ T cells and $\alpha_4\beta_7^+$ B cells [4,159]. Thus, the vitamin A metabolite retinoic acid appears to be a key molecules for controlling lymphocyte homing to the intestines.

Mucosal dendritic cells

Expression of $\alpha_4\beta_7$ and CCR9 molecules on activated T cells is specifically induced by MLN-DCs, PP-DCs or lamina propria DCs, but not by PLN-DCs or splenic DCs [168,169,189]. Additionally, these GALT-DCs are very efficient at inducing $FoxP3^+$ Treg cells, while splenic DCs are unable to induce Treg cells [133,135,138]. As described above, induction of gut-homing molecules and $FoxP3^+$ expressing Treg cells requires retinoic acid and thus DCs associated with the mucosal immune system must be especially well equipped to steer these processes. Indeed, GALT-DCs were shown to express vitamin A metabolizing RALDH enzymes [4] and are thus able to produce retinoic acid (Figure 5). The enzyme RALDH2 has been described to be the most abundantly expressed vitamin A converting enzyme in MLN-DCs [133,190], while PP-DCs highly express RALDH1[4]. And since splenic and PLN-DCs only display very low expression levels of these enzymes [4,133,191], RALDH expression seems to be a special feature of DCs that are associated with the mucosal immune system.
To identify which DC subset within gut-associated lymphoid tissues is responsible for regulation of gut-tropic T cells, DCs were sorted from PP and MLN based on the expression of conventional DC markers CD8, B220 and CD11b. Yet all DC

subsets were found to induce gut-homing molecules $\alpha_4\beta_7$ and CCR9 on T cells [168,185]. This would suggest that all DCs within gut-associated lymphoid tissues are equally efficient at generating gut-tropic T cells. However, recently a distinct subset of DCs, based on the expression of CD103, has been identified in murine MLNs that represent ~50% of total MLN-DCs and are more efficient at inducing gut-tropic T cells compared to their $CD103^-$ counterparts [192,193]. These DCs express RALDH enzymes at higher levels when compared to $CD103^-$ MLN-DCs and are better at inducing retinoic acid receptor-dependent signalling in T cells [133,188]. Consequently, induction of gut-homing molecule expression on activated T cells and $FoxP3^+$ Treg cell differentiation by $CD103^+$ MLN-DCs is enhanced when compared to T cell priming by $CD103^-$ MLN-DCs [133,192,193]. A $CD103^+$ DC subset with similar characteristics has also been identified in human MLNs [194,195].

Also a $CD103^+$ DC subset resides within the small intestinal lamina propria that displays similar properties as the $CD103^+$ DCs in MLNs [138]. This suggests that the $CD103^+$ MLN-DCs represent a population of migratory DCs derived from the intestinal lamina propria. Studies have shown that indeed $CD103^+$ DCs can be detected in lymph vessels draining the mesenteries [196] and can transport orally derived antigen from the intestine to the draining MLN. Within these LNs, $CD103^+$ DCs will induce gut-tropic T cells and regulatory T cells upon their activation [138,193,195]. That indeed $CD103^+$ MLN-DCs are derived from the intestinal lamina propria was shown in $CCR7^{-/-}$ mice [193]. CCR7 is required for DC migration from tissues to draining LNs and in the absence of CCR7, the numbers of $CD103^+$ DCs were strongly reduced in the MLN.

Within the mucosal immune system additional cells express RALDH enzymes. Intestinal epithelial cells lining the gut are known to highly express RALDH1 [4,197] and these cells have been shown to produce retinoic acid from dietary vitamin A *in vitro* [198-200]. Furthermore, stromal cells from MLNs express retinoic acid metabolizing enzymes, and *in vitro* cultured MLN stromal cells can directly induce gut-homing molecule expression on activated T cells [191,201]. Thus, within the intestines and GALT, multiple cell types exist that express vitamin A metabolizing enzymes and thus produce retinoic acid, which is crucial for the maintenance and functioning of the mucosal microenvironment.

3.3 Differentiation of mucosal DCs

As described above, GALT-DCs, in particular the $CD103^+$ subset, are able to produce retinoic acid, which enables them to preferentially promote Th2 differentiation [202-204], gut-homing molecule expression and Treg cell conversion [133,135,138,168,189,194]. Therefore, RALDH expression by GALT-DCs is crucial for their retinoic acid producing capacity. The differential expression of RALDH enzymes by GALT-DCs, and not by splenic or PLN-DCs,

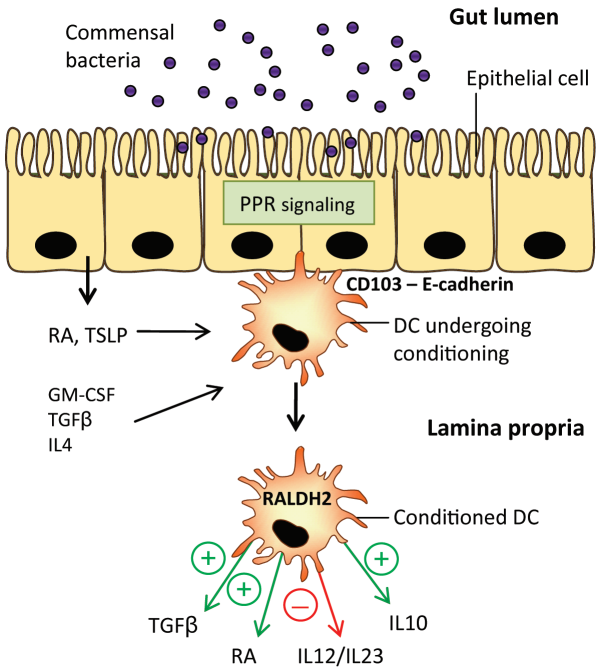

Figure 6. DC conditioning by the mucosal microenvironment.

The functional properties of intestinal dendritic cells (DCs) are imprinted within the intestinal environment. Epithelial cells (ECs) are ideal candidates to condition mucosal DCs. These cells express E-cadherin on the basolateral surface of the cell [205, 206], which is the ligand for CD103 expressed by most mucosal DCs. Expression of these molecules allows cell-cell contact between mucosal DCs and intestinal epithelial cells. Intestinal epithelial cells, but possibly also other cell types present within the intestines, produce imprinting factors for the differentiation of mucosal DCs from blood-derived precursors. Retinoic acid (RA), transforming growth factor-β (TGFβ), thymic stromal lymphopoietin (TSLP), IL-10, as well as GM-CSF, IL-13, and IL-4 are imprinting factors involved in conditioning of these DCs. These factors condition DCs to express RALDH2 enzymes. Conditioned DCs downregulate IL-12 and IL-23 production and enhance production of RA, IL-10 and TGFβ. In addition, signals through PRRs expressed by DCs and intestinal epithelial cells possibly contribute to mucosal imprinting of $CD103^+$ lamina propria DCs by inducing RALDH enzyme expression in mucosal DCs or the production of imprinting factors by intestinal epithelial cells. Conditioned $CD103^+$ lamina propria DCs preferentially drive Th2-type and regulatory T cell responses and efficiently induce gut-homing molecules on lymphocytes.

suggests a role for the mucosal environment in the induction and regulation of RALDH enzyme expression by DCs. Epithelial cells (ECs) are ideal candidates to educate mucosal DCs. Intestinal epithelial cells express E-cadherin, which is the ligand for CD103, on the basolateral surface of the cell [205,206]. This may allow cell-cell contact between intestinal DCs and the epithelial cells (Figure 6). In fact, CD103 has been reported to be induced under the influence of TGFβ, which can be produced by epithelial cells [207,208]. And thus, CD103 expression by DCs in the intestinal lamina propria could therefore be seen as a signature of their interaction

with intestinal epithelial cells. Furthermore, *in vitro* experiments showed that contact with gut epithelial cells induces CD103 expression and RALDH enzyme expression in bone marrow- and monocyte-derived DCs. In addition, intestinal epithelial cells educated DCs to induce Th2 cell polarization as well as gut-homing molecule expression on activated T cells [94,194,204,209]. Also, conditioning by intestinal epithelial cells promoted DCs to drive the development of $Foxp3^+$ Treg cells [94,194]. Factors that have been described to influence mucosal DC differentiation and that might be produced by intestinal epithelial cells, but possibly also by other cell types present within the intestines, are retinoic acid, TGFβ, thymic stromal lymphopoietin (TSLP), IL-10, as well as GM-CSF, IL-13, and IL-4. Of these factors, retinoic acid and GM-CSF plus IL-4 have been shown to induce RALDH expression in DCs (Figure 6).

A similar tissue-specific imprinting mechanism by peripheral tissue cells was shown for the skin. In coculture with dermal fibroblasts, DCs were imprinted to induce the expression of the skin-specific homing molecule E-selectin ligand on activated T cells [209]. Thus, it is thought that tissue-derived DCs, which develop from circulating DC precursors, acquire their tissue-specific imprinting capacity after having entered the tissue microenvironment.

In addition to the exposure to intestinal imprinting factors, DCs come in contact with pathogens present within the intestinal lumen via their pattern recognition receptors (PRRs). This interaction may additionally influence the mucosal phenotype of DCs in the intestinal lamina propria. It has been shown with ***in vitro*** stimulated bone marrow-derived DCs that RALDH2 mRNA expression increases upon TLR5 stimulation with flagellin or upon TLR2 stimulation with zymosan [160,210]. It is therefore possible that in the intestine also TLR-mediated signals or signals through other PPRs contribute to mucosal imprinting and RALDH expression in $CD103^+$ lamina propria DCs. The literature indicates, however, that DCs, which are exposed to intestinal factors, express lower TLR levels and are thus less well able to respond to TLR ligands [133,211-213].

3.4 Retinoic acid in lymph node development

It has been known for a long time that retinoic acid is an important player in embryonic organogenesis (reviewed in [214]). Recently, it has been demonstrated that retinoic acid also plays a role in lymph node development [215].

Development of secondary lymphoid organs like PPs and MLNs involves clustering of hematopoietic lymphoid tissue–inducer (LTi) cells, characterized as $CD4^+$ $CD3^-$ IL-$7R\alpha^+$, with $VCAM^+$ stromal organizer cells [216-218]. Subsequently, triggering of the lymphotoxin-β receptor (LTβR) on stromal organizer cells by lymphotoxin-αβ (LTαβ) on LTi cells [219] causes stromal organizer cells to synthesize chemokines and adhesion molecules that attract and retain more LTi cells [220]. This initiates a chain of events that involves further accumulation and signalling of LTi cells and

the organization of cell clusters to the complete development of the LN. Recently it was shown that the very first clustering of LTi cells and stromal organizer cells is controlled by the production of CXCL13 [215]. Retinoic acid was shown to be the inducing factor for CXCL13 expression in stromal cells and neurons adjacent to the location of lymph node development most likely are the providers of retinoic acid during LN development, because of their expression of RALDH enzymes [215]. These studies demonstrate that not only retinoic acid is crucial for mucosal immune homeostasis in adults, but also that retinoic acid is essential for the formation of the immune system during embryonic development.

The mucosal immune system must remain silent to harmless antigens while allowing vigorous immune responses towards harmful pathogens. It is now becoming clear that vitamin A plays a crucial role in maintaining immune homeostasis since the vitamin A metabolite retinoic acid is essential in multiple immunological process like the balance of Treg cells versus Th17 cells, epithelial barrier function, mucosal DC differention and gut-homing of T cells and IgA^+ B cells. In order to maintain the mucosal microenvironment and its immune balance, it is therefore of importance to learn how RALDH levels within the mucosal immune system are regulated and may be manipulated to correct dysregulation of immune homeostasis.

4. Thesis outline

Vitamin A plays an important role in maintaining mucosal immune homeostasis. Dysregulation of this balance, for instance by impaired vitamin A metabolism or vitamin A deficiency results in the development uncontrolled inflammatory conditions. The studies described in this thesis focus on the different cell types involved in maintainance of the mucosal microenvironment and immune balance. Furthermore, the effects of vitamin A deficiency and differences in vitamin A metabolism on the mucosal immune system in health and disease were addressed.

Chapter 2 describes the role of the LN microenvironment in providing signals for T cell tropism. Gut or skin draining LNs were transplanted into the popliteal fossa, so that DCs entering these LNs are bringing in antigen from the same peripheral site. These transplantations show that the microenvironment of mucosal MLNs and not peripheral LNs (PLNs), supports the induction of $\alpha_4\beta_7$, but not CCR9, on T cells upon their activation. Furthermore, MLN stromal cells showed expression of vitamin A converting enzymes allowing production of retinoic acid by these cells. While in vitro cultured MLN stromal cells were able to induce gut-homing tropism on activated T cells directly, addition of RALDH-low unpulsed bone marrow-derived DCs (BM-DCs) strongly enhanced the expression of gut-homing molecules $\alpha_4\beta_7$ and CCR9. These results demonstrate a crucial role for MLN stromal cells

in creating an instructive mucosal microenvironment in lymph nodes and that stromal cells, DCs and lymphocytes cooperate for efficient differential imprinting of tissue tropism.

Chapter 3 deals with the capacity of the peripheral tissue microenvironment to imprint DCs in such a way that they can induce skin and small intestine homing receptors on activated T cells. Upon activation by antigen-pulsed BM-DCs, $CD8^+$ T cells up-regulated the skin homing receptor E-selectin ligand when co-cultured with dermal fibroblasts and gut homing receptors CCR9 and $\alpha_4\beta_7$ when co-cultured with small intestinal epithelial cells. Soluble factors, such as retinoic acid, as well as cell-cell contact were essential for the induced tissue tropism imprinting capacity of DCs. This shows that peripheral tissue stromal and epithelial cells produce factors that license DC to induce tissue-specific homing receptors and thereby transmit information about their tissue of origin and the site of antigen capture to T cells.

In **chapter 4**, we have investigated how the expression of RALDH enzymes in MLN-DCs and MLN stromal cells is regulated postnatally. Studies with Trif mutant and MyD88-/- animals demonstrated that expression and activity of RALDH enzymes in MLN-DCs is independent of TLR signalling. On the contrary, dietary vitamin A appeared to be crucial for RALDH expression in MLN-DCs and MLN stromal cells. Furthermore, retinoic acid directly regulated the level of RALDH expression in BM-DCs as well as lymph node stromal cells, thereby regulating RALDH expression within the mucosal immune system and consequently maintaining mucosal immune homeostasis. These data establish that dietary vitamin A plays a crucial role in proper functioning of the mucosal immune system.

C57BL/6 and BALB/c mice are known as prototypical Th1- and Th2-type mice respectively, and retinoic acid has been described to skew T cells upon their activation towards the Th2-type profile. In **chapter 5** of this thesis, we therefore investigated whether C57BL/6 and BALB/c mice differ in their capacity to produce retinoic acid. We demonstrated that BALB/c mice expressed higher levels of RALDH enzymes and had more retinoic acid-mediated signalling in the intestines. Consequently, MLN-DCs displayed higher RALDH activity, which led to increased induction of gut-homing molecule expression on $CD4^+$ T cells and $FoxP3^+$ regulatory T cells. The enhanced capacity to induce gut-homing molecules correlated with an increased accumulation of T cells and B cells in BALB/c small intestines when compared to C57BL/6 small intestines. Also, secretion of IgA into the lumen of the small intestines was higher in BALB/c mice when compared to C57BL/6 mice. Thus, these studies showed that the enhanced ability to convert vitamin A results in a better developed mucosal immune system.

In **chapter 6**, we investigated whether the differences in retinoic acid production and retinoic acid receptor signalling observed in BALB/c and C57BL/6 mice could be correlated with the severity of inflammatory disease such as colitis. BALB/c mice suffered from less severe DSS colitis compared to C57BL/6 mice and recovered more quickly. During colitis, BALB/c mice showed an increased ability to form tertiary lymphoid tissue, which could contribute to IgA production and increased numbers of regulatory T cells. Retinoic acid has numerous beneficial effects on the mucosal immune system implicating that an increase in retinoic acid signalling could potentially improve the outcome of inflammatory disease and recovery from inflammation in BALB/c mice compared to C57BL/6 mice.

Chapter 7 describes the investigation of LTi differentiation during LN development. We hypothesized that retinoic acid affects hematopoietic LTi differentiation in the embryo and demonstrated that oral supplementation of pregnant mothers with retinoic acid skewed the differentiation of hematopoietic precursors towards the final LTi phenotype. Consistently, administration of a vitamin A deficient diet to pregnant mothers led to a significant decrease of the LTi differentiation state in the developing LN of the embryo. Also, embryos from BALB/c mice, which displayed enhanced vitamin A metabolism when compared to C57BL/6 mice, had more mature LTi cells in their MLNs and adult BALB/c mice displayed larger mucosal lymphoid organs. Thus, retinoic acid is involved in local differentiation of LTi cells during LN development and we propose that vitamin A levels in the mother have a significant effect on the amount of LTi cells and the formation of LNs in the embryo.

Finally, in **chapter 8**, the findings described in this thesis are summarized and discussed in the context of recent developments in the research on vitamin A and the mucosal immune system. Potential future research directions are indicated, which may resolve unanswered questions. This will give further insight in the mechanism of how the mucosal immune system operates and how it can be influenced.

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2

Lymph node stromal cells and dendritic cells synergize for the efficient induction of gut-homing receptors on T cells

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2

Abstract

T cells are imprinted to express tissue-specific homing receptors upon activation in tissue-draining lymph nodes, resulting in their migration to the site of antigen entry. Expression of gut-homing molecules $\alpha_4\beta_7$ and CCR9 is induced by retinoic acid, a vitamin A metabolite produced by retinal dehydrogenases, which are specifically expressed in dendritic cells as well as stromal cells in mucosa-draining lymph nodes. Here, we demonstrate that mesenteric lymph node (MLN) stromal cell-derived retinoic acid can directly induce the expression of gut-homing molecules on proliferating T cells, a process strongly enhanced by DCs. Therefore, cooperation of sessile LN stromal cells with mobile dendritic cells warrants the imprinting of tissue specific homing receptors on activated T cells.

Introduction

Upon activation by dendritic cells (DCs), coming from either peripheral or mucosal sites, T cells are induced to express adhesion molecules and chemokine receptors to ensure that activated T cells will preferentially migrate to the site where DCs initially encountered the antigen [1-5]. For migration to the intestines, T cells require expression of integrin $\alpha_4\beta_7$ and chemokine receptor CCR9, although the requirement for CCR9 may not be absolute [6-11]. The ligand for $\alpha_4\beta_7$, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), is expressed in the lamina propria of the colon and small intestine [12-14], while CCL25, the ligand for CCR9, is mainly produced by epithelial cells of the small intestine [15-20].

In recent years more insight into the mechanisms by which homing receptors are induced has been obtained and an important role for the vitamin A metabolite retinoic acid (RA) in the upregulation of $\alpha_4\beta_7$ and CCR9 on activated T and B cells has been described [6,21]. Vitamin A (retinol) is first reversibly oxidized by alcohol dehydrogenases to form retinal, which in turn is irreversibly metabolized to RA by three members of the aldehyde dehydrogenase gene family, the retinal dehydrogenases 1-3 (RALDH1-3) [22,23]. In mice, RALDH1 through 3 are differentially expressed in DCs from gut-draining lymphoid tissues, forming the basis for the RA-induced gut tropism [6]. This differential expression suggests a role for the mucosal environment in the induction of RALDH enzymes. This regulation could occur at the site where antigen is initially captured by DCs, for example the intestinal epithelium and lamina propria, as suggested by several reports [4,24-26]. In addition, it can be envisaged that the microenvironment of the draining mucosal LNs, where the interaction and activation of lymphocytes takes place, also provides the appropriate signals for induction of T cell tropism, as shown in a recent paper by Hammerschmidt et al. [27]. Such a role can further be inferred from our earlier experiments in which we showed with transplantation studies that mucosa-draining cervical LNs (CLNs) are unique in their capability to induce mucosa-associated immune tolerance, as peripheral, non-mucosal, LNs (PLNs) transplanted to the site of CLNs were not able to induce immune tolerance [28].

To elucidate the role of the LN microenvironment in providing signals for T cell tropism, either gut or skin draining LNs were transplanted into a peripheral site, the popliteal fossa, so that DCs entering these LNs are bringing in antigen from the periphery. These transplantations show that the microenvironment of mucosal mesenteric LNs (MLNs) and not peripheral LNs (PLNs), supports the induction of $\alpha_4\beta_7$, but not CCR9, on T cells upon their activation. Furthermore, MLN stromal cells showed expression of vitamin A converting enzymes allowing production of RA by these cells. While *in vitro* cultured MLN stromal cells were able to induce gut-homing tropism on activated T cells directly, addition of RALDH-low unpulsed bone marrow-derived DCs (BM-DCs) strongly enhanced the expression of gut-homing molecules $\alpha_4\beta_7$ and CCR9. Altogether, our data demonstrates a crucial

role for MLN stromal cells in creating an instructive mucosal microenvironment in which three obligatory parties, the stromal cells, DCs and lymphocytes cooperate for efficient differential imprinting of tissue tropism.

2

Materials and Methods

Mice

BALB/c and C57BL/6 mice aged 8 to 12 weeks were purchased from Charles River (Sulzfeld, Germany) and DO11.10, β-actin-GFP/C57BL/6, MHC-II$^{-/-}$, OT-I, and OT-II transgenic mice and C57BL/6-CD45.1 and C57BL/6-CD45.2 congenic mice aged 6 to 8 weeks were bred at our own facilities. The Animal Experiments Committee of the VU Medical Center approved all of the experiments described in this study.

Transplantation of LNs to the popliteal site

Transplantation of donor MLNs (mesenteric) or PLNs (from axial, brachial or inguinal sites) to the popliteal fossa was performed as described before [29].

T cell enrichment, CFSE labelling, transfer and antigenic stimulation

Spleens and LNs from DO11.10, OT-I, OT-II or C57BL/6 mice were minced through a 100-μm gauze to obtain single cell suspensions. To deplete erythrocytes from spleen cell suspension, cells were incubated for 2 minutes on ice in lysis buffer (150 mM NH_4, 1 mM $NaHCO_3$, pH 7.4). $CD4^+$ or $CD8^+$ T cells were enriched to at least 60% and 85% respectively, using the CD4 or CD8 negative selection kit (Dynal, Oslo, Norway). Cells were labelled with 5 μM of 5,6-carboxy-succinimidyl-fluoresceine-ester (CFSE, Molecular Probes, Invitrogen, Breda, The Netherlands) at $3x10^7$ cells/ml for 10 min at 37° C. Cells were used for *in vitro* cultures with stromal cells and/or DCs or used for *in vivo* intravenous injections. Transplanted and control BALB/c or C57BL/6 mice were injected with approximately 10^7 OVA-specific T cells and were subsequently stimulated 24 hours later by intramuscular (i.m.) or intragastric (i.g.) administration of 200 μg OVA in 10 μl saline or 50 mg OVA in 200 μl saline, respectively. After antigenic stimulation, transplanted LNs were isolated and used as single cell suspensions for FACS analysis.

Immunofluorescence and flow cytometry

Single cell suspensions were made by cutting LNs with scissors, followed by digestion at 37ºC for 20 min, using Blendzyme 2 (Roche, Penzberg, Germany) and 100 U/ml DNAse I (Roche). Antibodies used were anti-ERTR7 (affinity purified from hybridoma cell culture supernatant), anti-CD4 (clone GK1.5, BD Pharmingen,

Woerden, The Netherlands), biotinylated anti-mouse DO11.10 TCR (KJ1-26, Caltag Laboratories, Burlingame, CA), PE-Cy7 conjugated anti-CD8 (eBiosciences), PE-Cy7 conjugated anti-CD4 (eBiosciences), PE conjugated anti-mouse OT-I/OT-II TCR (Vα2, eBiosciences), anti-$\alpha_4\beta_7$ integrin (clone DATK32, kindly provided by Dr. Alf Hamann, Charité Universitätsmedizin Berlin, Germany), rat anti-mouse CCR9 (clone 7E7), rat anti-mouse PNAd (clone Meca-79, kindly provided by Dr. E.C. Butcher, Stanford University), anti-B220 (clone 6B2), PE conjugated anti-CD11c (clone N418, eBioscience), anti-CD3 (clone KT3), Alexa Fluor 647 conjugated CD45 (clone MP33), biotin conjugated anti-MHC-II (clone M5/114) and 7-AAD (Molecular Probes, Invitrogen) or Sytox Blue (Invitrogen) to discriminate live versus dead cells. Secondary antibodies were Alexa conjugated goat anti-rat-IgG and Alexa conjugated streptavidin (Molecular Probes, Invitrogen).
Cells were analyzed with a FACScalibur (BD Biosciences, Breda, The Netherlands) or with a Cyan Advanced Digital Processing High-Performance Research flow cytometer (Beckman Coulter, Mijdrecht, The Netherlands). Cells were sorted using a MoFlo sorter (DakoCytomation, Glostrup, Denmark). Tissue stainings were analyzed on a Leica TCS-SP2-AOBS confocal laser-scanning microscope (Leica Microsystems Nederland BV, The Netherlands) and images were obtained with Leica confocal software.

In vitro experiments

To obtain stromal cells for *in vitro* cultures, MLNs and PLNs from C57BL/6 mice were dissociated with Blendzyme 2 as described above. $1x10^6$ or $4x10^6$ LN cells were grown per well of 96-wells or 24-wells flat bottom plates, respectively (Greiner Bio One, Alphen a/d Rijn, The Netherlands) in IMDM medium (Iscove's Modified Dulbecco's Medium from Gibco, N.Y USA) containing 10% fetal calf serum (FCS, Hyclone, Utah, USA), 50 µM β-mercaptoethanol (Merck, Darmstadt, Germany), 1% L-glutamine and 1% Penicillin-Streptomycin (Biowhittaker Europe, Verviers, Belgium) for 24 hours. Subsequently, nonadherent cells were removed, fresh medium was added and the culture was continued for one week.
Bone marrow was isolated from the femur and tibia using a mortar and cultured for one week in IMDM medium with 20 ng/ml GM-CSF to obtain BM-DCs. $1x10^5$ BM-DCs were cultured on stromal cells for 48 hours in a volume of 200 µl IMDM complete medium with 50 nM retinol (Fluka, Sigma-Aldrich, Zwijndrecht, The Netherlands). Subsequently, cells were FACS-sorted into MHC-II$^+$ CD11c$^+$ DCs and CD45$^-$ MHC-II$^-$ CD11c$^-$ stromal cells for RNA isolation.
$5x10^4$ purified C57BL/6 CD8$^+$ T cells were cultured on a layer of stromal cells with 50 nM retinol (Sigma-Aldrich), 10 µM citral (RALDH enzyme inhibitor, Sigma-Aldrich), 10 nM all-trans retinoic acid (RA, Sigma Aldrich), 1 µM LE135 (RA receptor inhibitor, Tocris Bioscience, Bristol, United Kingdom), and 1 µM LE540 (RA receptor inhibitor, Wako Chemicals, Neuss, Germany) as indicated and activated with $5x10^4$

CD3/CD28 T cell expander Dynabeads (Dynal, Invitrogen, Breda, The Netherlands) in presence or absence of $1x10^4$ unpulsed BM-DCs.
In all T cell activation assays, responding T cells were analyzed with flow cytometry after 96 hours.

RNA isolation, cDNA synthesis and real time PCR

Sorted MHC-II$^+$ CD11c$^+$ DCs, CD45$^-$ MHC-II$^-$ CD11c$^-$ LN stromal cells, and stromal cells after 7 days of culture were lysed in Trizol (Gibco BRL, Breda, The Netherlands) or RLT buffer (Qiagen Benelux, Venlo, The Netherlands). RNA was isolated by precipitation with isopropanol or by using the RNeasy kit (Qiagen Benelux) according to the manufacturer's protocol and cDNA was synthesized from total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Burlington, Canada) according to the manufacturer's protocol. RALDH1 (Aldh1A1), RALDH2 (Aldh1A2), and RALDH3 (Aldh1A3) specific primers and primers for housekeeping genes β-actin, Ubiquitin C, HPRT and GAPDH were designed across exon-intron boundaries using Primer Express software (PE Applied Biosystems, Foster City, CA). Real time PCR was performed on an ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems). Total volume of the reaction mixture was 10 μl, containing cDNA, 300 nM of each primer and SYBR Green Mastermix (PE Applied Biosystems). To correct for primer efficiency, a standard curve was generated for each primer set with cDNA from a pool of non-activated LNs. Expression levels of transcripts obtained with real time PCR were analyzed and normalized with geNORM v.3.4 software [30].

Statistics

Statistical analysis was conducted using 2 tailed Student's t-test for differences in mean fluorescence intensity (MFI), ratio of $\alpha_4\beta_7$ or CCR9 expression on activated T cells in LNs or cocultures, or differences in relative RALDH1, RALDH2, or RALDH3 mRNA expression levels in sorted DCs and stromal cells. Differences were considered significant when $p<0.05$ or $p<0.02$ as indicated.

Results

Donor-derived cells in transplanted LNs are stromal cells.

In earlier studies we showed by transplantation of LNs that differences exist between LNs with respect to their ability to allow the induction of mucosal tolerance [28]. Since DCs as well as lymphocytes are mobile cells that are likely to be replaced rapidly after transplantation these results suggested that the specific features that make up the differences between MLNs and PLNs reside within the stromal elements of the LN. To investigate this we transplanted MLNs from

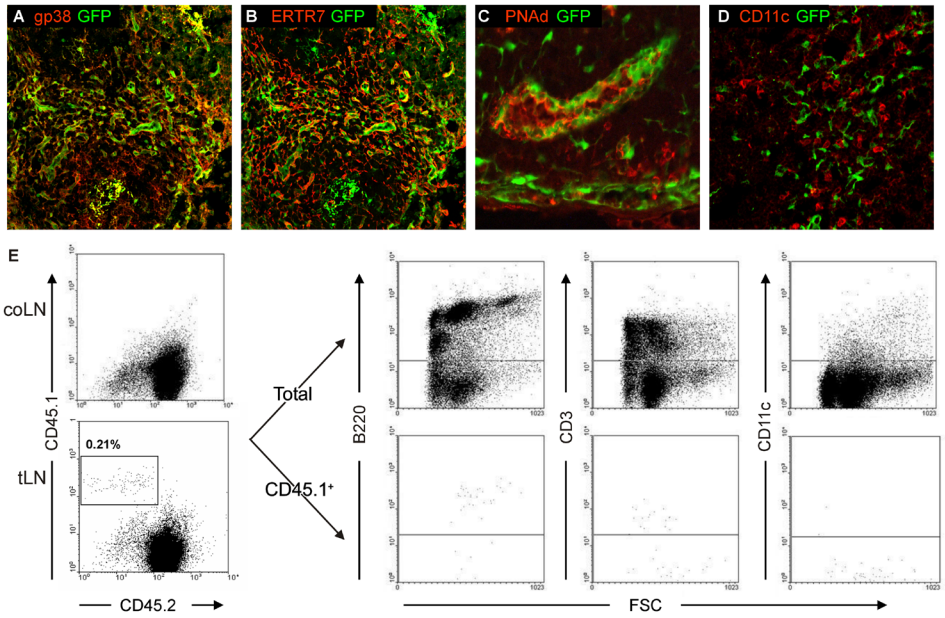

Figure 1. Non-haematopoietic cells remain in transplanted LNs, while all donor-derived haematopoietic cells disappear.

A-D, MLNs or PLNs from β-actin-GFP/C57BL/6 mice were transplanted into the popliteal fossa after removal of the popliteal LNs. Twelve weeks later, LNs were collected and stromal cells and haematopoietic cells in the transplanted LNs were analyzed with immunofluorescence for expression of (A) podoplanin (gp38, red), (B) ERTR7 (extracellular matrix marker, red), (C) PNAd (HEV marker, red), and (D) CD11c (DCs, red), while GFP$^+$ donor-derived cells appear in green in all pictures. Results shown are representative for 6 LNs transplanted for each group. E, MLNs from C57BL/6-CD45.1 mice were transplanted into the popliteal fossa of C57BL/6-CD45.2 recipients and collected after 5 weeks for FACS analysis of haematopoietic cells present in the transplanted MLN (tLN) or orthotopic PLN (coLN). The expression of B220, CD3 and CD11c on CD45.1$^+$ cells and all (total) haematopoietic cells is shown for transplanted MLN (tLN). Results were comparable to transplanted PLN. Results shown are representative for 5 LNs transplanted for each group.

β-actin-GFP/C57BL/6 mice into the popliteal fossa of C57BL/6 mice and addressed which cells were donor-derived at 12 weeks after transplantation. By staining for podoplanin (gp38) expressed on fibroblastic reticular cells (FRCs) and pericytes and for ERTR7, an extracellular matrix (ECM) glycoprotein produced by these cells, we could demonstrate that most podoplanin$^+$ cells which co-localized with ERTR7$^+$ ECM structures were GFP expressing donor-derived cells (Figure 1A, B), thus confirming that FRCs were donor-derived as reported for LN transplantations into gut mesenteries [27]. The majority of the high endothelial venules (HEVs) expressing PNAd (detected with MECA79) were of donor origin as well (Figure 1C). Staining for haematopoietic cells revealed that CD4$^+$ and CD8$^+$ T cells, B cells, as well as DCs were lacking GFP expression and thus were host derived

(Figure 1D and data not shown). To further prove that indeed haematopoietic cells were derived from the host, we transplanted LNs from CD45.1 mice into CD45.2 congenic hosts and observed that 5 weeks after transplantation very few donor-derived hematopoietic CD45.1$^+$ cells, predominantly B cells, could be detected in the transplanted LNs (Figure 1E).

In conclusion, we established that after transplantation of lymph nodes virtually all haematopoietic cells were derived from the host, while the stromal cells were donor-derived.

Activation of T cells in transplanted MLNs results in increased expression of $\alpha_4\beta_7$ but not CCR9.

To study the mechanism of tissue tropism induction guided by stromal cells *in vivo*, independent of mucosal tissue drainage, MLNs and PLNs were transplanted to the popliteal fossa of C57BL/6 mice. At twelve weeks after transplantation, C57BL/6 mice were injected with CFSE-labelled ovalbumin (OVA) specific transgenic CD8$^+$ Vα2$^+$ T cells (OT-I cells) and immunized with OVA in the sural muscle 24 hours later. At 72 hours after antigen administration, expression of mucosal homing molecules $\alpha_4\beta_7$ and CCR9 was analyzed on proliferating OT-I cells by flow cytometry. We observed a significant difference between expression of $\alpha_4\beta_7$ on OT-I cells activated in transplanted MLNs (tMLNs) versus transplanted PLNs (tPLNs) (Figure 2A). Induced expression in tMLN was comparable to levels on activated OT-I cells in the orthotopic MLNs (coMLN) upon intragastric administration of OVA (Figure 2A). No difference in $\alpha_4\beta_7$ expression was visible on the CFSE-labelled non-proliferating T cell populations in tMLN versus tPLN, indicative of a uniform entry of injected cells in both types of transplanted LNs and the necessity of T cell activation for indcution of this gut-homing molecule. Remarkably, the mucosal homing receptor CCR9 was not induced on proliferating OT-I cells in tMLNs, while its expression was readily induced in the orthotopic MLNs upon intragastric administration of OVA (Figure 2B). Identical results were obtained when transplanted mice were injected with CFSE-labelled OVA specific CD4$^+$ Vα2$^+$ T cells (OT-II cells). To rule out strain-specific effects the transplantations were also performed in the BALB/c mouse strain in combination with the DO11.10 transgenic T cells, giving similar results on the induction of $\alpha_4\beta_7$ expression (not shown). To exclude the possibility that donor-derived mucosal DCs might still be present after transplantation and interfere with T cell activation in the transplanted LNs, LNs from MHC-II deficient mice were transplanted to the popliteal fossa of C57BL/6 mice. These experiments showed similar induction of $\alpha_4\beta_7$ integrin on activated OT-II cells as observed in wild-type transplanted LNs (Figure 2A). Together, these results indicate that the mucosal microenvironment in the transplanted MLNs is partially preserved and allows for the induction of the gut-homing molecule $\alpha_4\beta_7$, but not CCR9, by host-derived DCs on antigen-specific T cells. For the induction of CCR9 on activated T cells additional factors might be required, presumably derived from the intestines

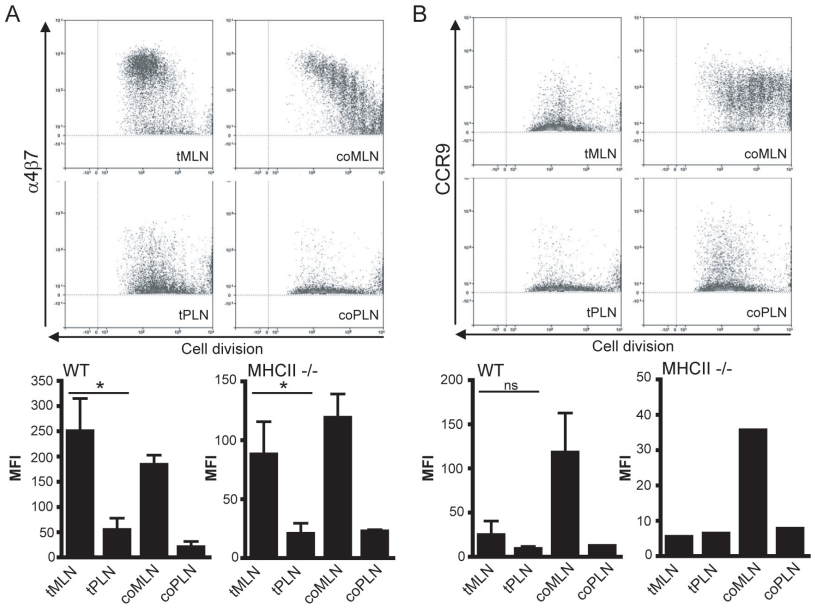

Figure 2. Transplanted MLNs provide a microenvironment for induction of $\alpha_4\beta_7$ integrin on stimulated antigen-specific T cells.

A and *B,* C57BL/6 mice were transplanted with PLN (tPLN) or MLN (tMLN) from C57BL/6 (WT) or MHCII$^{-/-}$ donor mice and received CFSE-labelled OVA-specific CD8$^+$ OT-I cells or CD4$^+$ OT-II cells respectively at 12 weeks after transplantation. Activated OT-I cells or OT-II cells in tMLNs, tPLNs, and orthotopic PLNs (coPLN) were analyzed by flow cytometry 3 days after immunization with OVA antigen in the sural muscle and activated OT-I or OT-II cells in orthotopic MLNs (coMLN) were analyzed after intragastric OVA immunization. Shown are flow cytometry plots of cell proliferation and expression of $\alpha_4\beta_7$ (*A, top*) and CCR9 (*B, top*) by proliferating OT-I cells in coLNs and WT tLNs. Data represent MFI ± SD of $\alpha_4\beta_7$ expression (*A, bottom*) and CCR9 expression (*B, bottom*) on activated OT-I cells in WT tLN and activated OT-II cells in MHCII$^{-/-}$ tLN. For each transplanted group 3 LNs were analyzed, while in each experiment one MLN and two popliteal LNs served as controls. Experiments were performed three times. Significant differences between tMLN and tPLN ($p<0.02$) are indicated by *.

and present within the intestinal lamina propria as well as in the lymph draining from the gut into the MLNs.

RALDH enzymes are expressed in mucosal DCs and MLN stromal cells.

Since the induction of gut-homing molecules on T cells has been described to be dependent on RA [6], we analyzed mRNA expression levels of RA producing RALDH enzymes in sorted CD45$^+$ MHC-II$^+$ CD11c$^+$ DCs versus CD45$^-$ stromal cells. As expected, sorted DCs from MLN (MLN-DCs) expressed high levels of RALDH2 as well as RALDH3, as shown before [6], while PLN-DCs expressed RALDH2 at much lower levels (Figure 3A). Notably, RALDH2 and RALDH3 were found to be expressed by freshly sorted CD45$^-$ stromal cells from MLNs, but not by PLN stromal cells (Figure 3C). Expression of RALDH1 was barely detectable in both PLN

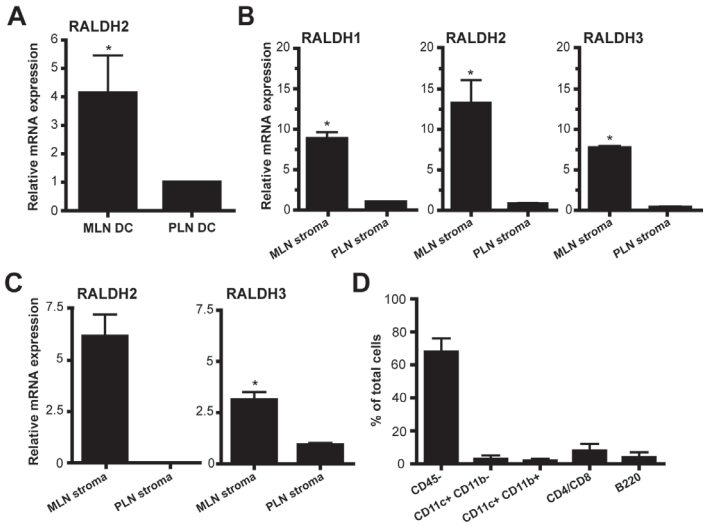

Figure 3. RALDH enzymes are expressed in MLN stromal cells and MLN DCs.

RNA was isolated from indicated cell types. Relative expression levels of RALDH1, RALDH2, and RALDH3 enzymes were measured by real time PCR. Expression of transcripts was normalized to endogenous references genes using geNORM v.3.4 software. Relative expression levels in PLN-DCs (A) and PLN stroma (B, C) was set at 1,0. Significant differences ($p<0.02$) are indicated by *. A, $CD11c^+$ MHC-II^+ DCs were FACS-sorted from PLNs and MLNs from 6 C57BL/6 mice of age 5-9 weeks, analyzed for RALDH1, RALDH2, and RALDH3 mRNA expression levels and normalized to GAPDH and HPRT. RALDH1 and RALDH3 expression were not detectable in these cells. The experiment was performed three times. B, 7 days cultured MLN stromal cells and PLN stromal cells were analyzed for relative expression levels of RALDH1, RALDH2, and RALDH3 enzymes and normalized to GAPDH and HPRT. The experiment was performed seven times. C, $CD45^-$ MLN stromal cells and PLN stromal cells, FACS sorted from freshly isolated MLN and PLN, were analyzed for relative expression levels of RALDH1, RALDH2, and RALDH3 enzymes and normalized to GAPDH and Ubiquitin C. RALDH1 expression was not detectable in these cells. RALDH2 was not detectable in PLN stroma. The experiment was performed three times. D, After 7 days of culture, LN stromal cells were trypsinized and analyzed with flow cytometry for expression of CD45, CD11c, CD11b, CD4, CD8 and 6B2. Shown are percentages of the total cell suspension. 3 wells were pooled for analysis and experiment was performed 4 times.

and MLN stroma (not shown). Therefore, MLN stromal cells may contribute to the generation of gut-homing T cells by producing RA, either by directly influencing T cells as suggested before [27], through instruction of DCs or by affecting both.

Gut-homing molecules are induced on activated T cells in the presence of MLN stromal cells and BM-DCs in vitro.

To see whether indeed stromal cells are instrumental to an instructive environment for induction of T cell tropism, we set up an *in vitro* assay to address this question. Since the low number of freshly sorted stromal cells would limit such *in vitro* studies, we addressed whether MLN stromal cells maintained RALDH levels after 7 days of culture. Indeed, RALDH1, RALDH2 and RALDH3 expression

was detected in cultured MLN stromal cells, and at very low levels in cultured PLN stromal cells (Figure 3B). Flow cytometric analysis of these stromal cultures showed that the majority of the cells were CD45-negative. Only a small percentage of haematopoietic cells was present after 7 days of culture (Figure 3D).

To investigate the effect of LN stromal cells on the induction of T cell tropism, stromal cells from PLNs versus MLNs were cultured for 7 days, after which OVA peptide-loaded BM-DCs and CFSE-labelled OT-I cells were added. Although all our cultures were performed in the presence of 10% FCS, which should account for approximately 40 nM retinol, experiments were carried out in the presence or absence of additional retinol to circumvent potential loss of bioactivity of FCS-derived retinol. After 96 hours, expression of mucosal homing molecules $\alpha_4\beta_7$ and CCR9 on proliferating antigen-specific T cells was analyzed by flow cytometry. When OT-I cells were stimulated in the presence of MLN stromal cells, the ratio of $\alpha_4\beta_7^+$ to $\alpha_4\beta_7^-$ T cells increased with each cell division upon addition of retinol, suggesting that RA production from retinol was involved in the induction of $\alpha_4\beta_7$ expression (Figure 4A). This effect was not seen with stromal cells from PLNs, where a stable ratio of $\alpha_4\beta_7^+$ to $\alpha_4\beta_7^-$ T cells was found. Similar results were found for the induction of CCR9. Only in the presence of MLN stromal cells and retinol, a robust expression of CCR9 on dividing T cells was seen (Figure 4B). Addition of retinol to the PLN stromal cell cultures allowed a slight induction of CCR9 on activated T cells, however not to the levels seen on T cells activated on MLN stromal cells. These results suggest that only in the presence of MLN stromal cells, but not PLN stromal cells, peptide-pulsed BM-DCs can strongly induce $\alpha_4\beta_7$ and CCR9 expression on activated T cells and that this process requires the addition of retinol, which is converted to RA by RALDH enzymes.

BM-DCs are not induced to express RALDH enzymes in vitro.

Since we have observed that BM-DCs, which were cultured together with small intestinal epithelial cells, were induced to express RALDH enzymes [25], we reasoned that a similar instruction could be mediated by MLN stromal cells. Therefore BM-DCs were co-cultured with MLN stromal cells or PLN stromal cells for 48 hours, after which stromal cells and DCs were sorted and analyzed for RALDH2 mRNA expression levels. BM-DCs cultured with PLN stromal cells express very low levels of RALDH2 mRNA, either in the presence or absence of added retinol. Moreover, MLN stromal cells were unable to further induce RALDH2 mRNA levels in BM-DCs, while MLN stromal cells expressed high levels of RALDH2 (Figure 4C). Therefore, in the *in vitro* cultures that allow the induction of gut-homing molecules on activated T cells, MLN stromal cells are the main source of RALDH enzymes, which are needed to convert retinol into RA.

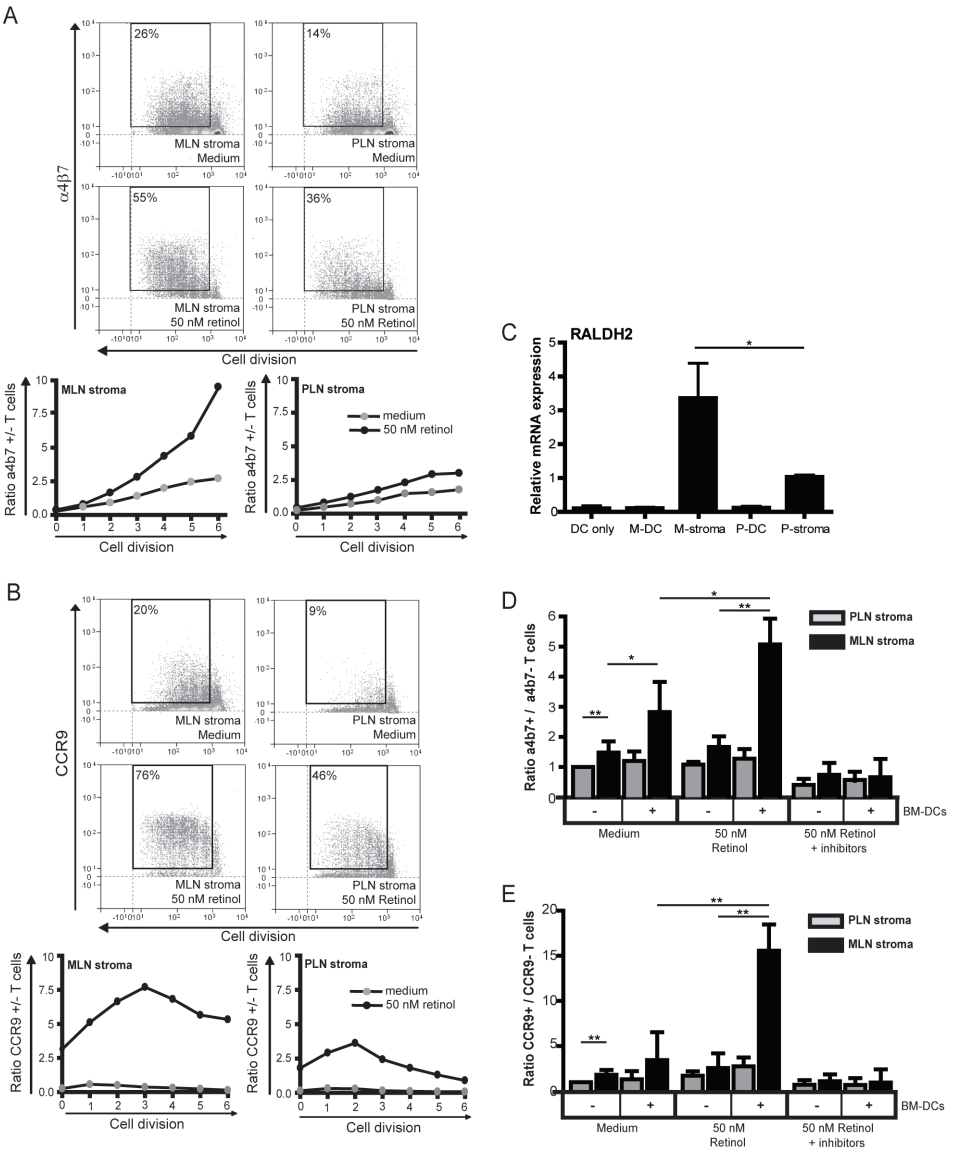

Figure 4. MLN stromal cells support induction of gut-homing molecules on activated CD8⁺ T cells, which is greatly enhanced by the presence of BM-DCs.

A and B, 1x10^5 CFSE-labelled OT-I cells were cultured in vitro with 5x10^4 OVA peptide-pulsed BM-DCs on a layer of MLN or PLN stromal cells in absence or presence of 50 nM retinol. After 96 hours, activated OT-I cells were analyzed by FACS for the expression of gut-homing molecules $\alpha_4\beta_7$ (A) and CCR9 (B). Representative FACS plots are shown. Boxes indicate percentage of $\alpha_4\beta_7$ or CCR9 expressing dividing T cells of total cell suspension. Experiment was performed two times. Data are calculated as the ratio of $\alpha_4\beta_7^+$ to $\alpha_4\beta_7^-$ (A, right column) and CCR9$^+$ to CCR9$^-$ (B, right column) activated antigen-specific T cells upon cell division on MLN or PLN stroma in absence (grey dots) or presence (black dots) of 50 nM retinol. C, 1x10^5 BM-DCs per well were cultured on a layer of MLN stromal cells or PLN stromal cells in presence of 50 nM retinol for 48 hours. Per

RALDH-low BM-DCs greatly enhance induction of gut-homing molecules by MLN stromal cells.

To address whether indeed stromal cells alone are sufficient for the induction of gut-homing molecules on T cells, $CD8^+$ T cells were activated with anti-CD3 and anti-CD28 coated beads on LN stromal cells in the absence or presence of BM-DCs. Retinol was either added as a substrate for RA production by stromal cells or omitted from the cultures. After 96 hours, $\alpha_4\beta_7$ and CCR9 expression on activated $CD8^+$ T cells was determined. When $CD8^+$ T cells were stimulated on MLN stromal cells in the absence of BM-DCs and retinol, the ratio of $\alpha_4\beta_7^+$ to $\alpha_4\beta_7^-$ T cells was significantly higher when compared to T cells activated on PLN stromal cells. Addition of retinol slightly increased the ratio of $\alpha_4\beta_7^+$ to $\alpha_4\beta_7^-$ T cells when activated on MLN stromal cells (Figure 4D).

Notably, addition of unpulsed BM-DCs strongly increased the expression of $\alpha_4\beta_7$ on bead-activated $CD8^+$ T cells in MLN stromal co-cultures. This increased expression was already observed when no retinol was added, suggesting that BM-DCs have a synergizing effect on the induction of gut-homing molecules on T cells (Figure 4D). Increased expression levels were not seen in PLN stromal cocultures, where a stable ratio of $\alpha_4\beta_7^+$ to $\alpha_4\beta_7^-$ T cells was observed and thus did the low levels of RALDH in BM-DCs not contribute to this induction. Similarly, only in the presence of stromal cells from MLNs an increasing expression of CCR9 on activated T cells was seen upon addition of retinol, while this was not observed on PLN stromal cells. Also, addition of unpulsed BM-DCs increased the expression of CCR9 on activated T cells in presence of MLN stromal cells. Remarkably, the ratio of $CCR9^+$ to $CCR9^-$ activated T cells was greatly increased upon addition of retinol (Figure 4E). These results show that MLN stromal cells can support the generation of gut-homing T cells directly, however the presence of DCs makes this process much more efficient.

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condition 4 wells were pooled for FACS sorting. $CD45^+$ $CD11c^+$ $MHC\text{-}II^+$ DCs and $CD45^-$ stromal cells were sorted from the co-cultures. Relative mRNA expression levels of RALDH2 were measured by real time PCR. Expression of transcripts was normalized to HPRT and Ubiquitin C using geNORM v.3.4 software. Relative expression in PLN stroma was set at 1,0. The experiment was performed five times. D and E, $5x10^4$ $CD8^+$ T cells were cultured in vitro with $5x10^4$ T cell expander Dynabeads on a layer of MLN stromal cells (black bars) or PLN stromal cells (grey bars) in the absence or presence of unloaded BM-DCs as indicated. 50 nM retinol, 10 µM citral plus 1 µM of RA receptor β inhibitors LE540 and LE135 was added to the cultures as indicated. After 96 hours, activated $CD8^+$ T cells were analyzed by flow cytometry for the expression of gut-homing molecules $\alpha_4\beta_7$ and CCR9. Data represent the ratio of $\alpha_4\beta_7^+$ to $\alpha_4\beta_7^-$ (D) and $CCR9^+$ to $CCR9^-$ (E) of activated $CD8^+$ T cells. Per condition 2 wells were pooled for analysis. The experiments have been performed three times. *, $p<0.05$, **, $p<0.02$.

Discussion

We have demonstrated here that stromal cells within MLNs mediate the induction of gut tropism by influencing proliferating T cells directly, a process strongly enhanced by DCs. We were able to show that, in spite of the fact that a MLN was transplanted to a site where it drains the skin rather than the intestine, MLN stromal cells can still provide the necessary microenvironment for the induction of gut-homing molecule $\alpha_4\beta_7$ expression on T cells, regardless of antigen transport by DCs from the intestine. Our in vitro studies revealed that MLN stromal cells can directly induce expression of gut-homing molecules on T cells, supporting the recent publication by Hammerschmidt et al. [27]. However, our data extend their observation since we showed that in the presence of DCs the induction of these molecules was markedly increased, while these DCs failed to upregulate the expression of RALDH enzymes.

Since an intimate interaction between LN stromal cells and DCs has been observed by immunofluorescence as well as by intravital microscopy [31-33], we propose that activation of T cells by DCs within organized lymphoid tissues also involves LN stromal cells. We therefore conclude that activation of lymphocytes within organized lymphoid tissues should no longer be viewed as an interaction between antigen presenting cells and lymphocytes, but rather as a response that involves three obligatory parties, namely stromal cells, DCs and lymphocytes.

Within the MLNs, $CD103^+$ DCs are able to induce the expression of both $\alpha_4\beta_7$ and CCR9, while $CD103^-$ DCs are only capable of inducing $\alpha_4\beta_7$ [34,35]. Since also $\alpha_4\beta_7^+$ $CCR9^-$ cells were shown to migrate to the intestines, both DC subsets can induce gut-homing tropism [9]. It has been suggested that $CD103^+$ DCs form the subset that constantly migrates from the intestinal lamina propria into the MLNs, while $CD103^-$ DCs enter the MLN via the blood [1,34-37]. In our experimental setting, the lamina propria-derived $CD103^+$ DCs are absent from the transplanted MLNs, suggesting that the absence of CCR9 expression on OVA-specific T cells is due to absence of this DC subset. However, in our in vitro cultures, LN-derived stromal cells by themselves are able to induce both $\alpha_4\beta_7$ and CCR9 expression on activated T cells, suggesting that stromal cells that have recently been removed from the intestinal environment are better equipped to induce gut-homing on T cells, possibly by producing higher amounts of RA. Although stromal cells within MLNs can directly induce gut-homing tropism on proliferating T cells, this process is markedly enhanced by DCs. And since DCs will initiate T cell activation by their presentation of antigen, we propose that stromal-derived RA might be transferred to and presented by DCs to T cells, perhaps through the immunological synapse formed between T cells and DCs during T cell activation. Compatible with this are the reported observations that uptake and presentation of RA to T cells can occur by DCs upon pre-incubation with RA. This process did not involve newly formed RA, since RALDH blockade did not affect induction of $\alpha_4\beta_7$ and CCR9

expression on T cells [38]. Similarly, a cell-cell interaction between MLN stromal cells and mucosal DCs might be required for the transfer of RA to mucosal DCs. Alternatively, DCs may somehow increase, by their interaction with MLN stromal cells, RALDH activity within MLN stromal cells without augmenting mRNA levels, resulting in the enhanced induction of gut tropism in T cells.

The stromal cells within LNs have been thought of as cells that simply provide a structure for immune cells to optimally interact with each other. However, recently it was shown that these stromal components also provide migratory guidance for T and B cells, while DCs have been reported to adhere to these cells [31,32]. In addition, stromal cells within the T cell area of LN, the fibroblastic reticular cells (FRCs), secrete extracellular matrix molecules as part of the conduit system, through which small size molecules can get rapid access to the LNs [33,39], while FRCs can mediate T cell survival through production of IL7 [40]. Furthermore, LN stromal cells can present endogenous antigen to T cells, hereby promoting peripheral tolerance induction [41,42]. Additionally, our studies have provided evidence that MLN stromal cells influence the final differentiation of T cells by differential expression of RALDH enzymes leading to the production of RA which creates an instructive mucosal microenvironment. Thus, our findings that unique stromal microenvironments exist in anatomically distinct LNs and that this may direct tissue-specific lymphocyte homing properties adds to the role stromal cells have in controlling immune responses.

Acknowledgements

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3

Instructive role of peripheral tissue cells in the imprinting of T cell homing receptor patterns

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3

Abstract

Tissue-specific homing of effector and memory T cells to skin and small intestine requires the imprinting of specific combinations of adhesion molecules and chemokine receptors by dendritic cells in the draining lymph nodes. Here, we demonstrate that CD8+ T cells activated by antigen-pulsed bone marrow-derived dendritic cells were induced to express the small intestine homing receptors $\alpha_4\beta_7$ integrin and chemokine receptor CCR9 in co-culture with small intestinal epithelial cells. In contrast, in co-culture with dermal fibroblasts the skin homing receptor E-selectin ligand was induced. Interestingly, the imprinting of gut homing receptors on anti-CD3/anti-CD28 stimulated T cells was induced by soluble factors produced by small intestinal epithelial cells. Retinoic acid was identified as a crucial factor. These findings show that peripheral tissue cells directly produce homing receptor imprinting factors and suggest that dendritic cells can acquire their imprinting potential already in the peripheral tissue of origin.

Introduction

Upon activation by dendritic cells (DC) in skin-draining lymph nodes, T cells up-regulate the skin homing receptors E- (E-lig) and P-selectin ligands (P-lig) as well as the chemokine receptors CCR4 and CCR10 [1-6]. Activation by DC from mesenteric lymph nodes (MLN) and Peyer's patches (PP) results in the gut-homing receptors $\alpha_4\beta_7$ integrin and CCR9 [4-9]. The role of lymph node resident DC versus DC that immigrated from peripheral tissues has not been addressed yet. DC from MLN and PP express retinal dehydrogenases (RALDH). They produce the vitamin A metabolite retinoic acid (RA) that induces up-regulation of $\alpha_4\beta_7$ and CCR9 [10]. Lamina propria-derived $CD103^+$ DC are responsible for the imprinting of gut homing receptors on T cells in PP and MLN [11,12]. Epidermal Langerhans cells (LC) were most efficient in the induction of skin homing receptors on $CD8^+$ T cells *in vitro* as compared to DC from skin-draining lymph nodes [4,6]. These findings suggest that the DC immigrating into draining lymph nodes from peripheral tissues, rather than the lymph node resident DC, are responsible for homing receptor imprinting. In support of this view, two independent homing phenotypes can be induced on T cells in the same lymph node by DC immigrated from different peripheral sites [13]. Moreover, the majority of DC in skin draining lymph nodes consists of immigrants, i.e. Langerhans cells and dermal DC [14].
Here, we have studied the role of the peripheral tissue microenvironment in the imprinting of skin and small intestine homing receptors by DC in co-culture systems. $CD8^+$ P14 T cells [15,16] upon activation with antigen-pulsed bone marrow-derived DC (BM-DC) up-regulated the skin homing receptor E-lig in co-culture with dermal fibroblasts or the gut homing receptors CCR9 and $\alpha_4\beta_7$ in co-culture with small intestinal epithelial cells (SIEC). Soluble factors such as RA induced the imprinting of the gut homing phenotype whereas cell-cell contact with dermal fibroblasts was important for the induction of E-lig on T cells. Our findings suggest that peripheral tissue stromal and epithelial cells produce factors that can directly induce homing receptors on T cells. These factors may license DC to also produce such factors and/or allow for the DC to shuttle these imprinting factors to the naive T cells in the regional draining lymph nodes.

Materials and Methods

Mice

C57BL/6 and TCR-transgenic Thy1.1 congenic P14 mice [15,16] were provided by the breeding facility of the University Medical Center Freiburg. All of the experimental procedures were in accordance with institutional, state and federal guidelines on animal welfare.

Media and chemicals

BM-DC, P14 cells and dermal fibroblasts were cultured in RP-10 [4]. Small intestinal epithelial cells (m-IC_{c12}) were cultured in m-IC medium as described [16].
The LCMV peptide GP33 (KAVYNFATM) was from Hermann GbR (Freiburg, Germany). All-trans RA was from Sigma-Aldrich (Munich, Germany). 9-cis RA and RA receptor antagonists (RAR, RXR26) were provided by Bernhard Homey.

Antibodies and flow cytometry

All antibodies were from BD Biosciences (Heidelberg, Germany) unless stated otherwise and used as FITC, PE or biotin conjugates. Biotin conjugated antibodies were revealed with PE-Cy5 conjugated streptavidin. Antibodies used here: anti-CD16/CD32 (FcγR II/III) (2.4G2), anti-CD90 (Thy1.1) (HIS51), anti-CD8α (53-6.7), anti-IA^b (AF6-120.1), anti-CD11c (HL3), anti-CD103 (M290), anti-$\alpha_4\beta_7$ (DATK32), E-selectin/human IgG-Fc-Chimera (R&D Systems, Wiesbaden, Germany), anti-hu-IgG-FITC (DakoCytomation, Hamburg, Germany), rat anti-CCR9 [18], kindly provided by Reinhold Förster (Hannover, Germany), mouse anti-rat IgG (H+L) (Jackson ImmunoResearch, Newmarket, UK). FACS staining was done as described [4,6]. Data were acquired and analyzed on a FACScan instrument using CellQuestPro software (BD Biosciences). Anti-pan TGFβ was purchased from R&D systems (Wiesbaden, Germany).

Isolation and preparation of cells

BM-DC were prepared as described [4] but without IL-4. m-IC_{c12} cells were differentiated for 6 days on collagen in 24-well plates and cultured as described previously [17]. Primary SIEC were isolated as described [19]. Dermal fibroblasts were isolated from the skin of 2 day old mice with 5 U/ml dispase (Invitrogen, Karlsruhe, Germany) for 1 h at 37°C. The dermis was separated from the epidermis, washed in PBS and incubated in collagenase I (500 U/ML) (Worthington Biochemical Corporation, Lakewood, USA) for 45 min at 37°C. Cells were singularized, washed in PBS and resuspended in medium. Medium was changed after 24 h and cells were cultured until confluent. P14 spleen cells were prepared as described [4,6]. Total splenocytes were used (referred to as P14 cells) or CD8α (Ly-2) MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany)-purified $CD8^+$ P14 T cells were used in some experiments to exclude bystander cell effects.

In vitro priming of T cells

BM-DC were harvested on day 7-9 and pulsed with 1 µM GP33 peptide (BM-DC-GP33) [4,6] or used unpulsed. $1x10^5$ BM-DC-GP33/well were incubated with P14 splenocytes ($2x10^5$/well) with or without m-IC_{c12} in 24-well plates (Greiner bio-one, Frickenhausen, Germany) for 4 days in a volume of 2 ml RP-10/m-IC medium (1:1 vol/vol). Retinoic acid receptor antagonists (8 µM RAR or 3 µM RXR) were added to

these co-cultures from the beginning. Co-cultures using BM-DC-GP33 ($1x10^4$/well) were incubated with $2x10^4$ splenocytes and $1x10^4$ dermal fibroblasts in round bottom 96-well plates (Corning Life Science, Wiesbaden, Germany) for 6 days in a total volume of 200 µl RP-10. Supernatants were collected at day 4 of co-culture and stored at -20°C. Cells were analysed by flow cytometry. Cell culture inserts (1 µm pore diameter; BD Falcon, Heidelberg, Germany) were used in transwell experiments. m-IC_{c12} or dermal fibroblasts were cultured at the bottom of 24-well plates, BM-DC-GP33 and P14 splenocytes were co-cultured in the cell culture inserts. Cell numbers were as described above.
Antibody activated splenocytes (soluble anti-CD3ε (145-2C11, 3 µg/ml) and anti-CD28 (37.51, 1.5 µg/ml) (BD Biosciences) were primed by addition of all-trans or 9-cis RA (10 nM and 7.5 µM, respectively).

Isolation of RNA and real time-PCR

Total RNA was isolated from BM-DC, m-IC_{c12} and from BM-DC co-cultured with m-IC_{c12} for 4 days using RNeasy Mini Kit *50* (Qiagen, Hilden, Germany). DC were re-isolated with CD11c MicroBeads using an AutoMACS following the manufacturer's instructions or have been seperated from m-IC_{c12} by transwell culture inserts. cDNA was prepared from 1 µg template RNA using Qiagen Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany). RALDH-1,-2 and -3 specific primers and primers for housekeeping gene 18S RNA were designed using Roche www.universalprobelibrary.com and were purchased from TIB Molbiol Syntheselabor GmbH (Berlin, Germany). The corresponding probes were obtained by Molecular Biochemicals (Mannheim, Germany). Real time PCR was performed on a Light Cycler 1.5 (Roche Molecular Biochemicals, Manheim, Germany) using LightCycler TaqMan Master (Roche Molecular Biochemicals, Mannheim, Germany). Total volume of reaction mixture was 20 µl, containing 1 µl cDNA, 4 µl Master Mix (TaqMan Master, see above) 1 µl of each primer (forward and reverse, end concentration 0.33 µM respectively), 1 µl probe in a final concentration of 0.1 µM and 12 µl RNase free water. A negative control was always included consisting of the same ingredients without any cDNA. The expression of transcripts was related to 18S RNA. CT values for 18S were subtracted from CT values for RALDH 1, 2 and 3, respectively (ΔCT) and normalized to values to ΔCT of DC alone (ΔΔCT). Fold increase was calculated by $2^{-(\Delta\Delta CT)}$.

Statistics and statistical analysis

Statistical analysis was conducted using Student's *t* test or ANOVA (non parametric) for the PCR data. Differences were statistically significant at $p<0.05$.

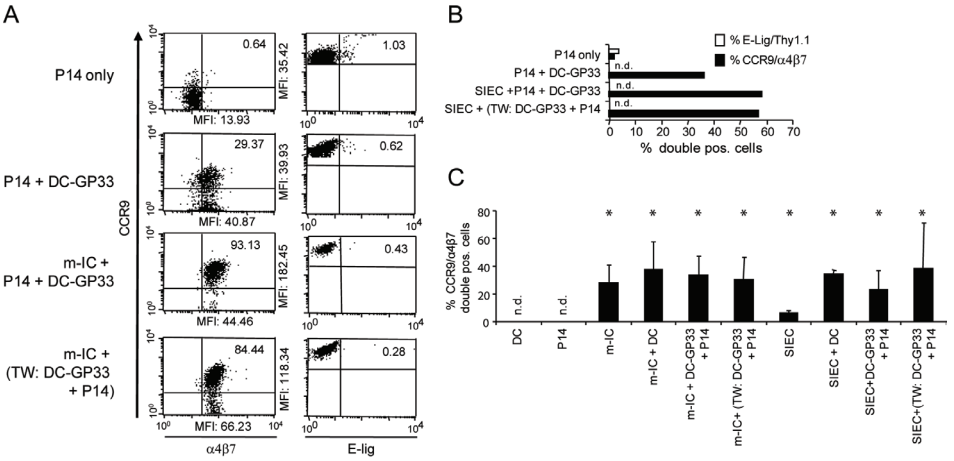

Figure 1: Induction of $\alpha_4\beta_7$ and CCR9 on activated CD8⁺ T cells in the presence of small intestinal epithelial cells.

(A) P14 splenocytes were cultured for 4 days alone (top), co-cultured with BM-DC-GP33 (second row), co-cultured with BM-DC-GP33 and SIEC (m-IC_{c12}) (third row) or co-cultured with BM-DC-GP33 and separated from m-IC_{c12} by using transwell culture inserts (bottom). Homing receptor induction on P14 cells was measured by flow cytometry. One representative of five independent experiments is shown. Gated on live Thy1.1⁺ cells. (B) Similar results were obtained with freshly isolated SIEC. Data are representative for three independent experiments. Gated on live Thy1.1⁺ cells. n.d.: not detected. (C) Supernatants from DC, P14 splenocytes, m-IC_{c12} or freshly isolated SIEC (co-)cultures were added as conditioned media (CM) to P14 cells activated with anti-CD3ε and anti-CD28. Live Thy1.1⁺ cells were analysed by FACS. Double positive cells ($\alpha_4\beta_7$/CCR9) were normalized to double positive splenocytes cultured without supernatants. Data represents the mean of three independent experiments. * = significant difference to CM from P14 cultures ($p<0.05$). TW: transwell. n.d.: not detected.

Results and Discussion

Small intestinal epithelial cells induce $\alpha_4\beta_7$ and CCR9 in T cell-BM-DC co-cultures by soluble factors.

It is conceivable that the antigen-loaded DC immigrating from peripheral tissues confer the information about their tissue of origin to T cells in local draining lymph nodes. In order to analyse the role of the peripheral tissue microenvironment in homing receptor imprinting, TCR transgenic P14 splenocytes (P14 cells) as a source for GP33 specific CD8⁺ T cells were co-cultured with BM-DC-GP33 in the presence or absence of the SIEC line m-IC_{C12}. Cells were analysed by flow cytometry on day 4 of co-culture (Figure 1A). Almost all CD8⁺ T cells expressed both $\alpha_4\beta_7$ integrin and CCR9 in the presence of m-IC_{c12} when compared to controls. A role for soluble factors was shown in transwell experiments separating co-cultures of P14 splenocytes and BM-DC-GP33 from m-IC_{c12} cells. The extent of homing

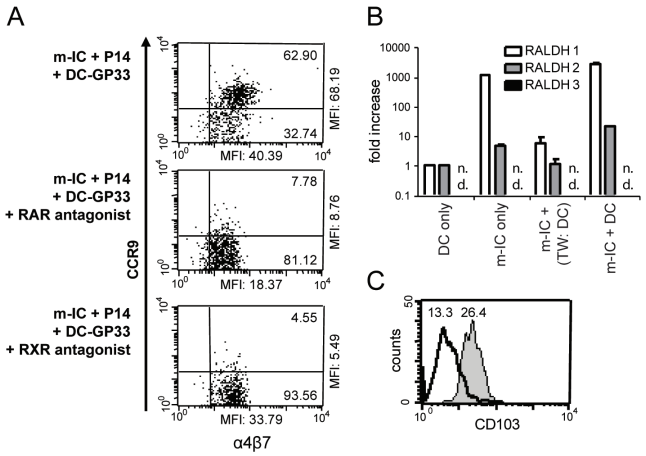

Figure 2: Effects of RA-receptor antagonists on the expression of gut homing receptors and induction of a gut-associated phenotype of DC by m-IC$_{c12}$.

(A) P14 splenocytes were co-cultured with BM-DC-GP33 and m-IC$_{c12}$ in the absence (top), or presence of RAR antagonist (middle), or RXR antagonist (bottom). Gated on live Thy1.1$^+$ cells. (B) Total RNA was isolated on day 4 of culture from BM-DC, mIC$_{c12}$, BM-DC from transwell (TW) cultures with mIC$_{c12}$ and from BM-DC previously co-cultured with m-IC$_{c12}$ re-isolated using CD11c MACS separation. cDNA was prepared and real time-PCR for RALDH1, 2 and 3 and the housekeeping gene 18S was performed in triplicates. n.d.: not detected. (C) Expression of CD103 on DC after co-culture with m-IC$_{c12}$. BM-DC were cultured in presence (shaded area) or absence of m-IC$_{c12}$ (bold line), harvested and stained at day 4. Cells were gated on CD11c/IA^b double positive cells. Histogram is representative for four independent experiments. Numbers indicate the mean fluorescence intensity (MFI).

receptor up-regulation was comparable to the cultures without transwells (Figure 1A, left column). E-lig was not detected (Figure 1A, right column). Similar results were obtained with freshly isolated primary SIEC (Figure 1B) using purified CD8$^+$ P14 T cells instead of splenocytes (data not shown).

Supernatants from co-cultures were used as conditioned medium (CM). P14 cells activated by anti-CD3ε and anti-CD28, and incubated with CM from co-cultures that contained either m-IC$_{c12}$ or primary SIEC significantly up-regulated $\alpha_4\beta_7$ integrin and CCR9 compared to CM from P14 cells only (Figure 1C). Interestingly, even CM from m-IC$_{c12}$ cells alone and to a lesser extent from primary SIEC alone were able to induce gut homing receptor expression on CD8$^+$ T cells (Figure 1C). These findings indicate that SIEC release soluble factors that can induce small intestine homing receptors on T cells. It remains to be determined whether the difference between primary SIEC alone and SIEC + DC (Figure 1C) is indicative of an imprinting of DC by SIEC.

Retinoic acid is involved in homing receptor imprinting in SIEC co-cultures.

Both isoforms of the vitamin A metabolite retinoic acid (RA), all-trans and 9-cis RA, efficiently induced $\alpha_4\beta_7$ integrin and CCR9 on P14 T cells activated with anti-CD3 and anti-CD28 (data not shown) as previously reported [10]. All-trans RA binds to RA receptor (RAR) only whereas 9-cis RA binds to RAR and RX receptor (RXR). Both receptors mainly function as heterodimeric, ligand-inducible transcription factors [20].

To test a potential role of RA, m-IC_{c12} were co-cultured with P14 cells and BM-DC-GP33 in the presence or absence of RA receptor antagonists. The upregulation of CCR9 was completely blocked in the co-cultures by both RAR and RXR antagonists whereas the expression level of $\alpha_4\beta_7$ was only reduced in presence of RAR antagonist (Figure 2A). In contrast to the antagonists used here, inhibition of RALDH by citral or of RAR by the antagonist LE135 efficiently suppresses RA- or MLN-DC-induced $\alpha_4\beta_7$ upregulation [10]. It has been reported that TGFβ is a potent regulator of $\alpha_4\beta_7$ [21]. Neutralization of TGF-β in the presence or absence of RAR and RXR ntagonists had no effect on the expression of $\alpha_4\beta_7$ in our co-cultures (supplementary figure S1). These results suggest that SIEC play an important role in the induction of gut homing receptors on T cells by production of imprinting factors such as RA. RA production by human intestinal epithelium has been reported [22] and seems to be crucial for gut homing receptor induction.

BM-DC show a gut-associated phenotype after co-culture with m-IC_{c12}.

$CD103^+$ DC isolated from MLN were the most potent in inducing a gut homing phenotype on T cells compared to CD103- MLN DC [11, 12]. They express RALDH enzymes and can produce the imprinting factor RA [10]. Furthermore, almost all lamina propria DC but only a subpopulation of DC from MLN express CD103 (αE chain of the integrin $\alpha_E\beta_7$). These $CD103^+$ lamina propria DC produce RA [23]. To evaluate whether BM-DC adopt a gut DC phenotype in the presence of SIEC *in vitro,* BM-DC were stained for CD11c, $I\text{-}A^b$ and CD103 before and after co-culture with m-IC_{c12}. At day 4 of co-culture, only a slight, but reproducible upregulation of CD103 could be detected compared to BM-DC cultured alone (Figure 2C). We also observed the induction of RALDH1 in DC after co-culture with m-IC_{c12} (Figure 2B). These data show a bias towards a gut-associated phenotype of the BM-DC induced by SIEC. This implies that DC may acquire the ability to produce RA in the tissue microenvironment of the lamina propria. Positive feedback loops for the production of RA have been defined [24,25]. Thus, interactions of DC with peripheral tissue cells may induce a differentiation program resulting in the tissue-specific phenotype of the DC [4-9]. It is also possible that DC shuttle soluble factors produced by peripheral tissue cells, in this case SIEC, to the lymph nodes as suggested for RA [21].

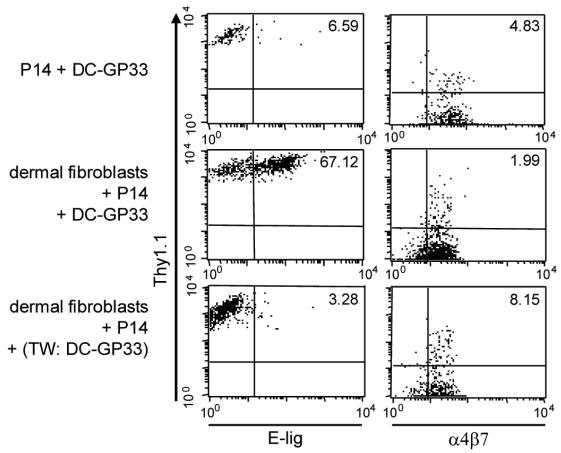

Figure 3. Induction of E-selectin ligand on DC-activated CD8⁺ T cells in the presence of dermal fibroblasts.

Expression levels of E-lig, a_4b_7 and CCR9 on P14 cells cultured with BM-DC-GP33 in the absence (top) or presence of dermal fibroblasts (middle). Dermal fibroblasts were separated from splenocytes co-cultured with BM-DC-GP33 by transwell culture inserts (TW, bottom). All plots are representative for three experiments. Gated on live Thy1.1⁺ cells.

BM-DC induce E-selectin ligands in the presence of dermal fibroblasts.

In order to test whether dermal fibroblasts from mouse skin have a similar impact on the imprinting of skin homing receptors on T cells, co-cultures with dermal fibroblasts, BM-DC-GP33 and P14 cells were set up and analyzed after 6 days. The skin homing receptor E-lig was induced in these co-cultures, in contrast to cultures without dermal fibroblasts (Figure 3, left column). Induction of skin homing receptors was not observed when cell culture inserts were used to separate DC and P14 cells from the fibroblasts (Figure 3, left bottom panel). These results suggest that cell-cell contact is necessary for the induction of skin homing receptors on T cells, although IL-12 can induce E-lig *in vitro* [26]. Most likely this cell interaction takes place between DC and skin cells as naive T cells do not have access to dermal fibroblasts before they are primed for skin homing in the lymph node *in vivo*. We did not see any effects on E-lig expression by the Vitamin D3 metabolite calcitriol (data not shown) which helps to induce CCR10 expression on human T cells [27]. CCR4 was upregulated in an activation-dependent manner (data not shown) [4]. Similar results were obtained in all of these settings when purified CD8 T cells were used instead of splenocytes, excluding bystander effects of non-T cells. Expression of the gut homing receptors $\alpha_4\beta_7$ integrin or CCR9 was not observed in these experiments (Figure 3, right column).

Our findings demonstrate an instructive role for peripheral tissue cells in the imprinting of skin and small intestine homing receptors on T cells. It has been

reported that stromal cells can influence DC differentiation [28-30] (reviewed in [31]). For Langerhans cells that differentiate in the skin from monocyte precursors [32, 33], a role for both contact-dependent mechanisms and soluble factors in the acquisition of tissue-specific characteristics has been described [34,35]. In summary, we suggest that the peripheral tissue microenvironment conditions DC to shuttle topographical information to the lymph node in a bimodal fashion. As reported, DC may themselves be induced to produce factors for homing receptor imprinting [10] and, in addition, may transport factors produced by peripheral tissue cells to the draining lymph nodes [21]. This would license the DC to induce tissue-specific homing receptors and thereby pass the information about their tissue of origin and the site of antigen entry on to the T cells.

Acknowledgements

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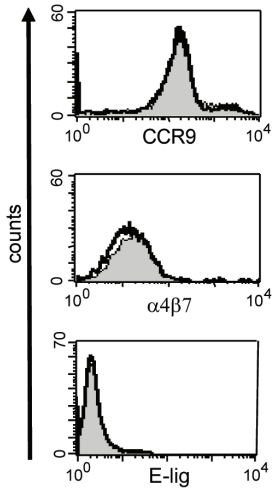
Supplemental data



Supplementary figure 1: Effect of TGF-β on homing receptor expression.

P14 splenocytes were co-cultured for 4 days with BM-DC-GP33 and m-IC_{c12} in the presence (bold line) or absence (shaded area) of 5 µg/ml anti-pan TGF-β antibodies and the expression levels of CCR9, $\alpha_4\beta_7$ and E-lig were analysed on Thy1.1$^+$CD8$^+$ T cells by flow cytometry. One representative of five independent experiments is shown.

4

Expression of RALDH enzymes in dendritic cells and stromal cells in gut draining lymph nodes is controlled by dietary vitamin A

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4

Abstract

The vitamin A metabolite retinoic acid (RA) plays a crucial role in mucosal immune responses. We demonstrate here that RA-producing RALDH enzymes are postnatally induced in mesenteric lymph node (MLN) dendritic cells (DCs) and MLN stromal cells. RALDH enzyme activity in migratory $CD103^+$ MLN-DCs did not depend on Toll-like receptor signalling. Remarkably, RA itself could directly induce RALDH2 in both DCs and stromal cells *in vitro*. Furthermore, using vitamin A deficient mice, it was found that RA-mediated signalling was strongly reduced within the small intestines, and that RALDH2 mRNA expression in both migratory $CD103^+$ MLN-DCs and MLN stromal cells was strongly diminished. Moreover, supply of vitamin A to vitamin A deficient mice restored RA-mediated signalling in the intestine and RALDH activity in migratory $CD103^+$ MLN-DCs. Our results show that RA-dependent signalling within the intestine is indispensable for RALDH activity in the draining MLN.

Introduction

Vitamin A has long been recognized for its role in immunity. It is currently estimated that 190 million preschool-aged children and 20 million pregnant women in developing countries are vitamin A deficient [1], leading to increased risk of night blindness and mortality [2,3]. Vitamin A deficiency compromises mucosal barriers of the respiratory and gastrointestinal tracts [4]. It has also been described that vitamin A deficiency affects the ability of macrophages and neutrophils to migrate to sites of infection, phagocytose and kill bacteria [4,5]. Vitamin A also plays a critical role in the development of adaptive immune responses in the intestines. Through expression of the vitamin A converting aldehyde dehydrogenase 1A (ALDH1A, also called retinaldehyde dehydrogenase (RALDH)) enzymes, dendritic cells (DCs) and stromal cells in gut-associated lymphoid organs, like Peyer's patches (PP) and mesenteric lymph nodes (MLN), are able to produce the active metabolite retinoic acid (RA) necessary to induce gut-homing specificity on activated T cells [6-11], FoxP3-expressing regulatory T (Treg) cells [12-18] and IgA producing B cells [19-21], and to suppress the differentiation of Th17 cells [13,16,17]. Therefore, RA appears to be a key molecule that controls lymphocyte homing properties and mucosal immune responses. It is thus of importance to know how RALDH levels are regulated within the mucosal immune system.

The major pathway of RA synthesis depends on two steps. Vitamin A is first reversibly oxidized by alcohol dehydrogenases (ADH) to form retinaldehyde. These enzymes are expressed in most cells including DCs. Next, retinaldehyde is irreversibly metabolized to RA by three members of the aldehyde dehydrogenase gene family, ALDH1A1 (RALDH1, ALDH1A2 (RALDH2) and ALDH1A3 (RALDH3) [22-24]. RALDH expression is limited to certain cell types, like intestinal epithelial cells [7,25-27], nerve fibers [28] and MLN stromal cells as well as DCs in PPs, MLNs and intestinal lamina propria, while splenic or peripheral lymph node (PLN)-DCs display only very low expression levels of these enzymes [6,7,10,12]. The differential expression of RALDH enzymes by DCs associated with mucosal tissues, suggests a role for the mucosal environment in the induction of these enzymes. It has not been fully established how and when intestinal DCs acquire the RA-producing capacity.

In MLNs, a distinct subset of DCs has been identified that express RALDH enzymes at high levels and are marked by the expression of CD103 [12]. These $CD103^+$ DCs are better equipped to induce retinoic acid receptor (RAR) signalling in T cells [29], $FoxP3^+$ Treg cell differentiation, and gut-homing receptor expression on activated T cells when compared to $CD103^-$ DCs [9,12]. $CD103^+$ MLN-DCs represent a lamina propria-derived migratory population, which acquire the mucosal phenotype within the intestinal environment [9,18,30-33]. Indeed, we [34] and others [35] have shown that contact of bone marrow-derived (BM)-DCs with gut epithelial cells induced expression of RALDH enzymes and educated BM-

DCs to induce gut-homing molecules on T cells *in vitro.* Similar data have been published for human DCs [36]. Factors involved in mucosal DC imprinting include RA, as well as GM-CSF, IL-13, TGFβ, TSLP and IL-4 [33,35-37]. Of these factors, RA and GM-CSF plus IL-4 have been described to contribute to RALDH expression in DCs [37].

In addition to contact with intestinal epithelial cells, DCs are in contact with microbes present in the intestinal lumen via pattern recognition receptors which include Toll like receptors (TLRs) [38,39], C-type lectin receptors [40] and intracellular Nod-like receptors [41]. Recently it was described that RALDH2 expression in both BM-DCs and splenic DCs was induced upon zymosan stimulation [42]. It is therefore possible that also in the intestine, TLR-mediated signals contribute to RALDH expression in $CD103^+$ lamina propria DCs.

It remains to be determined how RALDH expression is regulated in MLN stromal cells. However, RALDH expression by adult MLN stromal cells might represent an intrinsic capacity, since transplanted MLN retained expression of RALDH in a skin-draining location [10]. Also, PLN transplanted into the mesenteries maintained low RALDH levels compared to MLN, irrespective of intestinal-derived factors draining to these transplanted LN [6].

Here we show that RALDH enzymes are postnatally induced in MLN-DCs and MLN stromal cells suggesting that external factors during postnatal development are involved in maturation of the RALDH-dependent intestinal immune system. We found that expression and activity of RALDH enzymes is independent of TLR signalling, because MLN-DCs from Trif mutant, $MyD88^{-/-}$ and C57BL/6 WT animals exhibit similar RALDH activity. Remarkably, dietary vitamin A appeared to be crucial for RALDH expression in MLN-DCs and MLN stromal cells and loss of RALDH activity in mice that were deficient for vitamin A could be quickly restored by vitamin A supplementation. These data point to an essential role of dietary vitamin A for a proper functioning of the mucosal immune system.

Materials and Methods

Mice

C57BL/6 mice aged 1 to 14 weeks, $MyD88^{-/-}$ mice [43] and $Trif^{lps2/lps2}$ mice aged 10-14 weeks were bred at our own animal facilities and were housed under specific-pathogen-free conditions (SPF). In $Trif^{lps2/lps2}$ mice, the Trif protein is modified by a single base-pair deletion resulting in a dysfunctional protein [44]. The Animal Experiments Committee of the VU Medical Center approved all of the experiments described in this study.

Generation of Vitamin A-deficient, -control and –high mice

C57BL/6 mice obtained from Charles River (Charles River, Maastricht, The Netherlands) were mated and pregnant females either received a chemically defined diet that lacked vitamin A (the modified AIN-93M feed, MP Biomedical, Solon, Ohio, USA; vitamin A deficient (VAD)), contained vitamin A (2800 IU/kg in the modified AIN-93M feed, MP Biomedical; vitamin A control (VAC)) or contained double the amount of vitamin A (5600 IU/kg in the modified AIN-93M feed, MP Biomedical; vitamin A high (VAH)). These diets started at 8-9 days of gestation. The pups were weaned at 4 weeks of age and maintained on the same diet at least until 10 weeks of age before analysis was performed. All mice were housed under SPF conditions.

Cell sorting, ALDEFLUOR assay and flow cytometry

Single cell suspensions were made by cutting LNs with scissors, followed by digestion at 37ºC for 20 min, using 0.5 mg/ml Blendzyme 2 (Roche, Penzberg, Germany) and 0.2 mg/ml DNAse I (Roche) in PBS, while constantly stirring. Cell clumps were removed by pipetting the cells over a nylon mesh. LN cells were washed and resuspended in PBS with 2% NBCS.

For sorting of DCs and stromal cells, cells were stained with biotin conjugated anti-MHC-II (clone M5/114), PE conjugated anti-CD11c (clone N418, eBioscience, Immunosource, Halle-Zoersel, Belgium), Alexa fluor 647 (Invitrogen, Breda, The Netherlands) conjugated anti-CD45 (clone MP33) and 7AAD (Molecular Probes, Invitrogen) to discriminate between live versus dead cells. $CD45^+$ $MHC\text{-}II^+$ $CD11c^+$ DCs and $CD45^-$ $MHC\text{-}II^-$ $CD11c^-$ stromal cells were sorted using a MoFlo XDP cell sorter (Beckman Coulter, Woerden, The Netherlands).

RALDH activity in individual cells was measured using ALDEFLUOR staining kits (StemCell Technologies, Grenoble, France), according to the manufacturer's protocol with modifications. Briefly, cells suspended at 10^6 cells per ml in ALDEFLUOR assay buffer containing activated ALDEFLUOR substrate (365 nM) with or without the RALDH inhibitor diethylaminobenzaldehyde (DEAB, 7.5 µM) were incubated for 40 min at 37˚C. For flow cytometric analysis of ALDEFLUOR-reacted cells, cells were subsequently stained with PE conjugated anti-CD11c (clone N418, eBioscience), biotin conjugated anti-CD103 (clone M290, BD Bioscience, Breda, The Netherlands), Alexa fluor 647 conjugated anti-MHC-II (clone M5/114) and with Sytox Blue (Invitrogen) to discriminate between live versus dead cells. Secondary antibody used was PERCP conjugated (BD Bioscience) or PE-Cy7 conjugated streptavidin (eBioscience). Cells were analyzed with a Cyan ADP flow cytometer (Beckman Coulter).

In vitro experiments

Bone marrow was isolated from femurs and tibia using a mortar and cultured for one week in IMDM medium containing 10% FCS, 50 µM β-mercaptoethanol, 1% L-glutamine, 1% Penicillin-Streptomycin (IMDM complete medium) and 20 ng/ml GM-CSF (clone X63) to obtain bone marrow derived DCs (BM-DCs).

$1x10^5$ BM-DCs were cultured in 96-wells plates (Greiner Bio One, Alphen aan den Rijn, The Netherlands) for 24 hours in a volume of 200 µl IMDM complete medium in presence or absence of 100 nM all-trans retinoic acid (RA, Sigma-Aldrich, Zwijndrecht, The Netherlands). $1x10^6$ BM-DCs were cultured for 24 hours in a volume of 1 ml IMDM complete medium with anti CD40 antibody (8 µg/ml, clone 1C10), imiquimod (5 µg/ml, Cayla InvivoGen, Toulouse, France), LPS (1µg/ml, Sigma-Aldrich), PAM3CSK4 (100 ng/ml, Cayla InvivoGen), Phorbol 12-myristate 13-acetate (PMA, 100 ng/ml, Sigma-Aldrich), curdlan (33 µg/ml, Sigma-Aldrich), Polyinosinic–polycytidylic acid sodium salt (Poly I:C, 10 µg/ml, Sigma-Aldrich) or zymosan (5 µg/ml, Sigma-Aldrich).

Stromal cells from PLNs and MLNs were cultured in 96-wells plates (Greiner Bio One) for 7 days as described earlier [10] and incubated with 5 nM all trans-RA, 50 nM all trans-RA and 500 nM all trans-RA in a volume of 200 µl complete IMDM medium for 24 hours. Cells were lysed in RLT buffer from RNeasy kit (Qiagen Benelux, Venlo, The Netherlands) or lysis buffer from mRNA Capture kit (Roche, Woerden, The Netherlands) for RNA isolation and analysis with Real time PCR.

RNA isolation and real time PCR

RNA was isolated from sorted $CD45^+$ $MHC\text{-}II^+CD11c^+$ DCs, $CD45^-$ $MHC\text{-}II^-$ $CD11c^-$ LN stromal cells and stimulated BM-DCs and LN stromal cells using RNeasy kit (Qiagen Benelux) or mRNA Capture kit (Roche) according to the manufacturer's protocol. cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Burlington, Canada) or with the Reverse Transcription System (Promega Benelux, Leiden, The Netherlands), respectively. Primers for RALDH1 (*Aldh1A1)*, RALDH2 (*Aldh1A2*), RALDH3 (*Aldh1A3*), RARβ, GM-CSF *(Csf2)*, IL-4 and TGF-β1 and for housekeeping genes ubiquitin C, hprt, and GAPDH (Isogen Life Science, De Meern, Netherlands; Invitrogen) were designed across exon-intron boundaries using Primer Express software (PE Applied Biosystems, Foster City, CA). Real time PCR was performed on an ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems). Total volume of the reaction mixture was 10 µl, containing cDNA, 300 nM of each primer and SYBR Green Mastermix (PE Applied Biosystems). The comparative Ct method (ΔCt) was used to assess relative changes in mRNA levels between samples.

Statistics

Results are given as the mean ± SD. Statistical analyses were perfomed using the 2-tailed Student's *t* test.

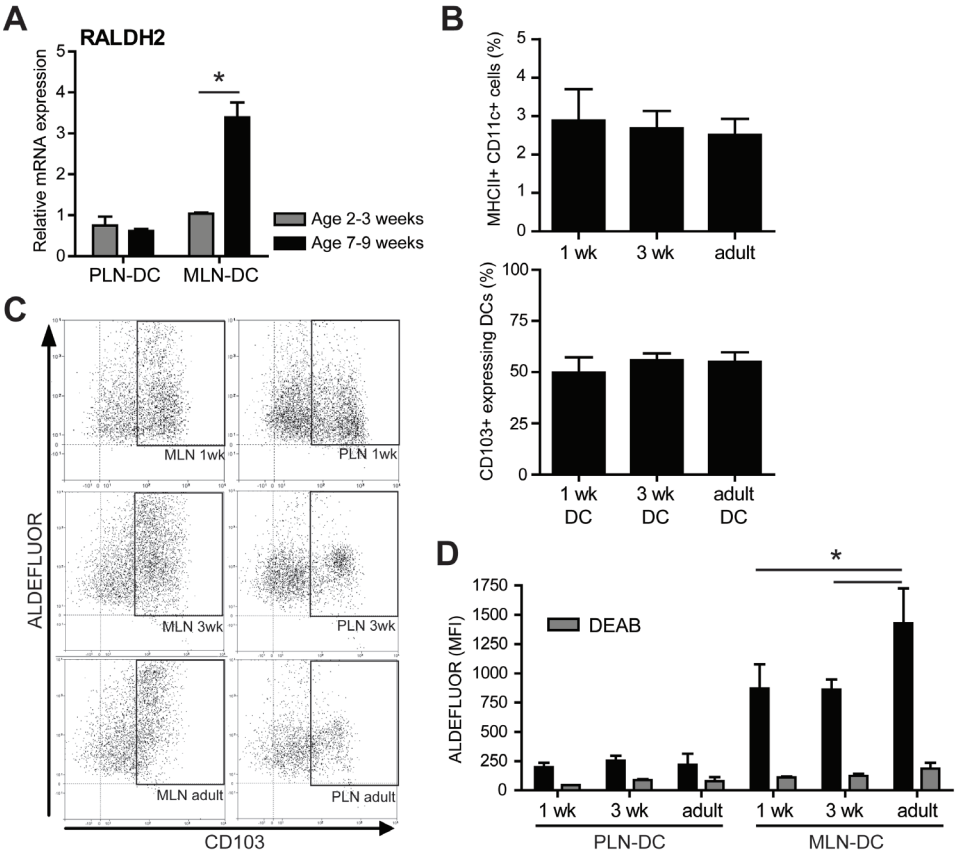

Figure 1. RALDH expression and activity in MLN-DCs is increased during postnatal development.

(A) CD45^{+} MHC-II^{+} CD11c^{high} DCs were FACS-sorted from MLN and PLN from female C57BL/6 mice aged 2-3 weeks and aged 7-9 weeks. Sorted DCs were analyzed for expression of RALDH1, RALDH2 and RALDH3 by real-time PCR. Relative expression levels in young PLN-DCs was set at 1. Per group 3-6 animals were used for FACS-sorting. Experiment was performed two times. RALDH1 expression was not detectable in these LN, while RALDH3 expression was too low to give any consistent difference. Significant difference is indicated by * ($p<0.01$). (B) MLN cell suspensions from C57BL/6 mice aged 1, 3, or 9 weeks were analyzed for MHC-II, CD11c and CD103 expression. Data represent percentage ± SD of MHC-II^{+} CD11c^{high} DCs of total LN cells (top) and percentage of MHC-II^{+} CD11c^{high} DCs expressing CD103 (bottom). Per group 6-7 animals were used for analysis. (C) CD103^{+} MHC-II^{+} CD11c^{high} DCs in LN cell suspensions from C57BL/6 mice aged 1, 3, or 9 weeks were analyzed for RALDH activity using ALDEFLUOR assay. Representative FACS plots are shown for ALDEFLUOR signal and CD103 expression by MHC-II^{+} CD11c^{high} DCs in PLN and MLN. Box indicates CD103 gate for calculations of ALDEFLUOR MFI shown in D. (D) Data represent ALDEFLUOR MFI ± SD in CD103^{+} LN-DCs in absence (black bars) or presence (grey bars) of RALDH inhibitor DEAB. Per group 7 animals were used for analysis. Significant differences are indicated by * ($p<0.01$). MLN, mesenteric lymph node; PLN, peripheral lymph node.

Results

RALDH expression and activity is increased during postnatal development.

The enzyme RALDH2 has been described to be the most abundantly expressed vitamin A converting enzyme in MLN-DCs and MLN stromal cells [6,10,12,37] and its expression is crucial for the induction of gut-homing T cells within adult MLNs. In order to determine how RALDH expression in these cells is controlled we first investigated at what point after birth MLN-DCs and MLN stromal cells started to express and showed functional activity of RALDH enzymes. Hereto, MHC-II$^+$ CD11c$^+$ DCs and CD45$^-$ MLN stromal cells were sorted from MLNs and PLNs from C57BL/6 mice, 2-3 weeks and 7-9 weeks of age and analyzed for RALDH2 mRNA expression. Remarkably, at an early age, MLN-DCs showed low expression levels of RALDH2 with levels that were similar as measured in PLN-DCs, while adult MLN-DCs highly expressed RALDH2 (Figure 1A). A similar age-related distribution was found for CD45$^-$ MLN stromal cells sorted from mice at the age of 2-3 weeks, which showed significantly lower expression of RALDH2 when compared to adult MLN stromal cells (Supplementary Figure 1). Notably, RALDH2 expression in PLN stromal cells was hardly detectable and expression of RALDH1 and RALDH3 mRNA was undetectable in all DC and stromal cell samples. In conclusion, RALDH2 expression in MLN-DCs and MLN stromal cells is postnatally induced over the course of 2-7 weeks.

Low expression of RALDH2 in MLN-DCs at the age of 2-3 weeks could simply be due to a failure of lamina propria-derived DCs to migrate to the draining MLN at this age. However, staining for CD103, which is now a well established marker for lamina propria-derived DCs [9,18,31], showed that already at 1 week after birth approximately 50% of MHC-II$^+$ CD11c$^+$ DCs expressed CD103 (Fig. 1B, bottom). Furthermore, the percentages of MHC-II$^+$ CD11c$^+$ DCs (Fig. 1B, top) and of CD103$^+$-expressing DCs (Fig. 1B, bottom) were not different between the different ages. This strongly suggests that already within the first week after birth lamina propria-derived DCs are migrating to MLNs and that RALDH levels must be lower in these DCs in the first weeks after birth.

To ensure that indeed mRNA for RALDH was translated into functional protein RALDH enzyme activity was analyzed in MLN cells from mice at different ages using the ALDEFLUOR assay. With this assay RALDH activity can be measured in individual cells by flow cytometry with a fluorescent substrate, specific for RALDH enzymes. Among MLN cells, RALDH activity was detected almost exclusively in CD103$^+$ MHCII$^+$ CD11c$^+$ cells of MLNs (Fig. 1C, left), corroborating that CD103$^+$ MLN-DCs have higher RALDH mRNA levels compared to CD103$^-$ DCs in MLNs [12]. Notably, we were not able to detect RALDH activity in MLN stromal cells, since the ALDEFLUOR assay was not suitable for stromal cell analysis. At 1 and 3 weeks of age, CD103$^+$ MLN-DCs showed significantly lower levels of RALDH activity than measured in adult CD103$^+$ MLN-DCs (Figure 1D). In addition, CD103$^+$

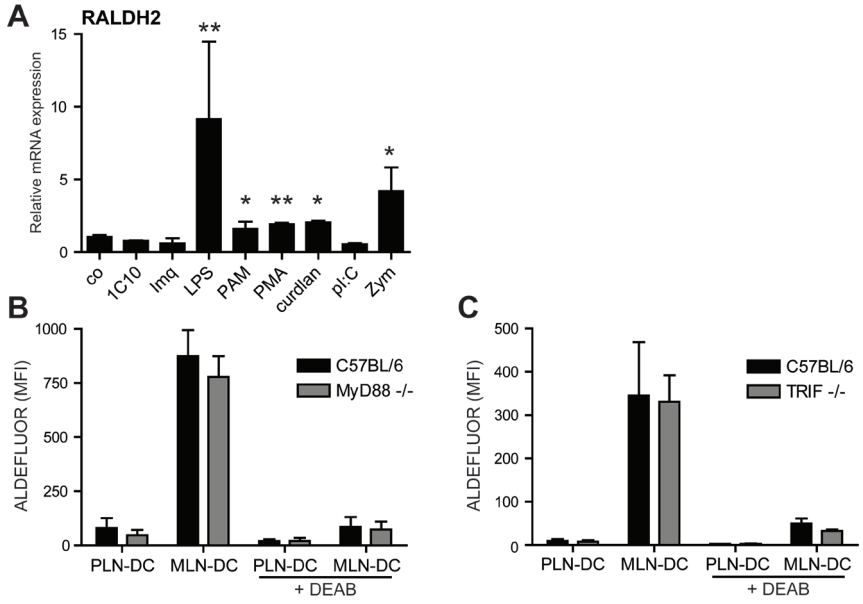

Figure 2. RALDH activity in MLN-DCs is independent of MyD88- and Trif-dependent signalling.
(A) BM-DCs from C57BL/6 mice were incubated with different TLR ligands for 24 hours. Relative mRNA expression levels of RALDH2 were measured by real time PCR. Relative expression in control BM-DCs (co) was set at 1. The experiment was performed 3 times. Significant differences are indicated by * ($p<0.05$) and ** ($p<0.02$). (B,C) MHC-II^{+} CD11c^{high} DCs in MLN and PLN cell suspensions from 10-14 week old C57BL/6, MyD88$^{-/-}$ mice and Trif mutant were analyzed for RALDH activity using ALDEFLUOR assay. Data represent ALDEFLUOR MFI ± SD in CD103^{+} MLN- and PLN-DCs in absence or presence of RALDH inhibitor DEAB for MyD88$^{-/-}$ animals (A) and Trif mutant animals (B) compared to C57BL/6. For each experiment 5 MyD88$^{-/-}$, 6 Trif mutant mice and 5 C57BL/6 mice were used.

PLN-DCs showed very low RALDH activity at all ages analyzed. In conclusion, not only RALDH expression, but also RALDH activity increased in MLN-DCs during postnatal development.

RALDH enzyme activity is not dependent on TLR signalling.

Next, we questioned how the expression and activity of RALDH is regulated during postnatal development. Mice are essentially born germ-free, but soon after birth, mucosal surfaces are colonized with high numbers of bacteria [45,46]. It is therefore conceivable that the induction of RALDH in MLN-DCs and MLN stromal cells is linked to postnatal colonization of the intestine by commensal bacteria. Also, recently it was described that both bone marrow derived DCs (BM-DCs) and splenic DCs were induced to express RALDH2 upon zymosan stimulation [42]. Indeed, we confirmed that RALDH2 expression was induced in zymosan-treated BM-DCs (Figure 2A), but we also observed a significant induction upon stimulation with PAM3CSK4, PMA, curdlan and LPS (Figure 2A). Since we and others [42] found

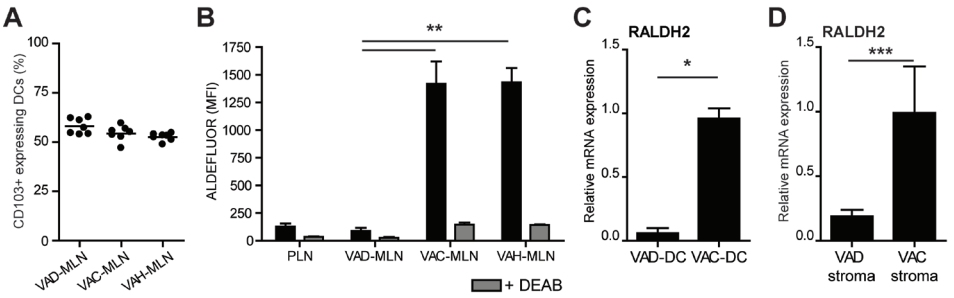

Figure 3. Dietary vitamin A regulates RALDH enzyme expression and activity in MLN-DCs.
(A,B) MHC-II^{+} CD11c^{high} DCs in MLN from C57BL/6 mice on vitamin A deficient (VAD), vitamin A control (VAC) or vitamin A high (VAH) diet aged 10 weeks were analyzed for CD103 expression and RALDH activity using ALDEFLUOR assay. Per group 7 animals were used for analysis. (A) Data represent percentage ± SD of MHC-II^{+} CD11c^{high} DCs expressing CD103 in individual MLNs. (B) Graphs show ALDEFLUOR MFI ± SD in CD103^{+} MLN-DCs and PLN-DCs from VAD, VAC and VAH mice in absence (black bars) or presence (grey bars) of RALDH inhibitor DEAB. (C,D) CD45^{+} MHC-II^{+} CD11c^{high} MLN-DCs and CD45^{-} MLN stromal cells were FACS-sorted from male VAD and VAC C57BL/6 mice, aged 11 weeks. Sorted cells were analyzed for expression of RALDH1, RALDH2 and RALDH3 by real-time PCR. Relative expression levels in VAC MLN-DCs (C) or in VAC MLN stromal cells (D) was set at 1. RALDH1 and RALDH3 expression were too low to give any consistent difference. Per group 6 animals were used for FACS-sorting. Experiment was performed two times. Significant differences are indicated by * ($p=0.01$), ** ($p<0.001$) and *** ($p=0.05$).

TLR-induced upregulation of RALDH2 expression in DCs *in vitro,* we analyzed that RALDH enzyme activity in MLN-DCs from MyD88$^{-/-}$ and Trif mutant mice. Analysis of MLN-DCs from MyD88$^{-/-}$ (Figure 2B) and Trif mutant mice (Figure 2C) showed that normal RALDH enzyme activity could be measured in these DCs, substantiating that TLR signalling via these adaptor proteins is not mandatory for induction of RALDH in MLN-DCs *in vivo* [47].

RALDH enzyme activity was abrogated in vitamin A deficient mice.

Since it has been shown that intestinal epithelial factors, among which the vitamin A metabolite retinoic acid (RA), induce RALDH expression in BM-DCs [35], we decided to address whether dietary vitamin A is crucial for the postnatal induction of RALDH enzyme activity. C57BL/6 mice were raised on a vitamin A deficient (VAD) and compared with animals raised on vitamin A control diet (VAC) or vitamin A high diet (VAH, containing double the amount of vitamin A). Serum retinol levels in 10 week-old VAC and VAH mice were 1.30 ± 0.01 μM and 1.43 ± 0.17 μM respectively, while serum retinol levels in VAD mice were undetectable at this age (detection limit 0.10 μM). Within the MLN, the proportion of DCs expressing CD103 was comparable in all 3 groups, showing that lamina propria-derived DCs were normally present in the MLNs (Figure 3A). Remarkably, the absence of dietary vitamin A almost abrogated RALDH enzyme activity, since ALDEFLUOR levels measured in MLN-DCs from VAD mice were reduced by 94% when compared

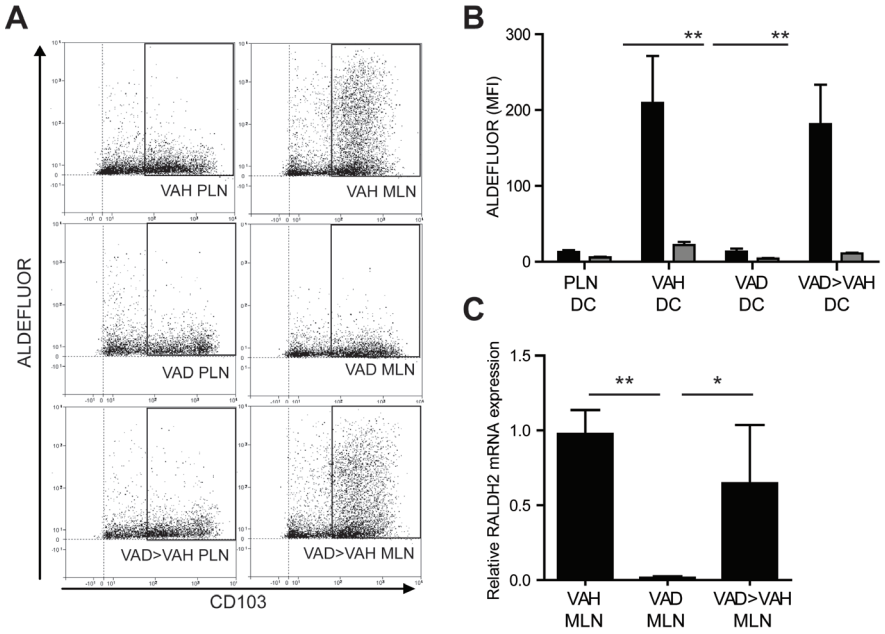

Figure 4. RALDH activity in VAD MLN-DCs is restored upon vitamin A supplementation.

MHC-II^{+} CD11c^{high} DCs in MLN cell suspensions from C57BL/6 mice, aged 12 weeks, on vitamin A deficient (VAD), vitamin A high (VAH) diet or from VAD mice that received VAH diet for 7 days (VAD>VAH) were analyzed for CD103 expression and RALDH activity using ALDEFLUOR assay. (A) Shown are representative flow cytometry plots of ALDEFLUOR activity and CD103 expression of MHC-II^{+} CD11c^{high} DCs from PLN and MLN. Box indicates CD103 gate for calculations of ALDEFLUOR MFI shown in B. (B) Graphs show ALDEFLUOR MFI ± SD in MLN-DCs from VAH, VAD and VAD > VAH mice in absence (black bars) or presence (grey bars) of RALDH inhibitor DEAB. Per group 8 animals were used for analysis. Experiment was performed two times. (C) RNA was isolated from MLN suspensions from VAH, VAD and VAD > VAH mice and analyzed for RALDH2 expression with real time PCR. Per group 3 MLNs were used for analysis. RALDH1 and RALDH3 expression were undetectable in these samples. Significant differences are indicated by * ($p=0.05$) and ** ($p<0.002$).

to MLN-DCs from VAC and VAH mice and comparable to levels observed in PLN-DCs (Figure 3B). In addition, RALDH enzyme activity in MLN-DCs from VAC and VAH mice was comparable, indicating that extra dietary vitamin A did not further increase RALDH enzyme activity in MLN-DCs (Figure 3B).

To evaluate whether dietary vitamin A also influenced RALDH mRNA expression, both CD45^{-} MLN stromal cells and MHC-II^{+} CD11c^{+} MLN-DCs were sorted from VAD and VAC mice and analyzed for RALDH2 mRNA expression. In agreement with our observation that RALDH enzyme activity in DCs required dietary vitamin A, RALDH2 mRNA expression levels in MLN-DCs from VAD mice were significantly reduced when compared to MLN-DCs from VAC mice (Figure 3C). In addition, CD45^{-} MLN stromal cells sorted from VAD mice also showed significantly lower levels of RALDH2 mRNA when compared to VAC mice (Figure 3B). In conclusion, dietary vitamin A is crucial for RALDH2 mRNA expression in both MLN-DCs and MLN stromal cells.

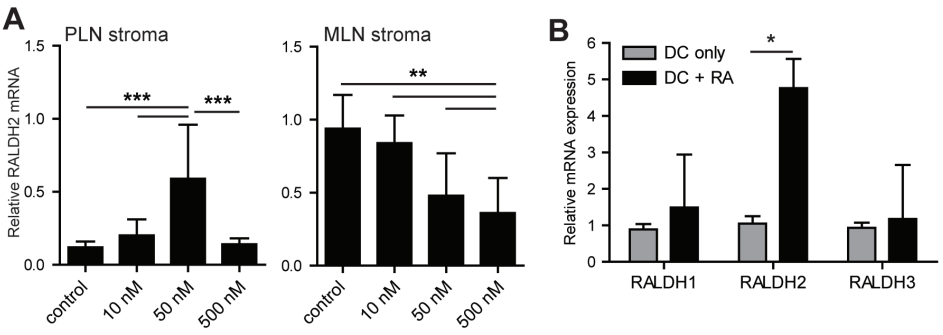

Figure 5. Regulation of RALDH expression in dendritic cells and LN stromal cells by RA.
(A) PLN and MLN stromal cells were cultured for 7 days and subsequently stimulated with 5 nM, 50 nM or 500 nM RA. After 24 hours, PLN stromal cells (A, left) and MLN stromal cells (A, right) were analyzed for RALDH2 expression with real time PCR. Relative expression levels in unstimulated MLN stromal cells (control) was set at 1. Experiment was performed 3 times. (B) BM-DCs were cultured alone or in presence of 100 nM RA. After 24 hours, BM-DCs were analyzed for expression of RALDH1, RALDH2 and RALDH3 by real-time PCR. Relative expression levels in control DCs (DC only) was set at 1 for each RALDH gene. Experiment was performed 4 times. Significant differences are indicated by * ($p<0.001$), ** ($p=0.01$) and *** ($p=0.05$).

Restoration of RALDH enzyme activity in MLN-DCs upon vitamin A supplementation.

As the lack of vitamin A in food abrogates RALDH activity in both MLN-DCs and MLN stromal cells, we questioned whether we could rescue this by feeding the VAD animals a vitamin A high (VAH) diet for 7 days (VAD>VAH). Surprisingly, supplementation with vitamin A resulted in a significant increase of ALDEFLUOR$^+$ CD103$^+$ MLN-DCs in these mice when compared to VAD animals (Figure 4A, B). Moreover, RALDH2 mRNA expression levels in total MLN, to which both DCs and stromal cells contribute, were increased upon vitamin A supplementation of VAD mice (Figure 4C). These results show that already after 7 days of vitamin A supplementation, the effects of vitamin A deficiency on RALDH enzymes were reverted and that MLN-DCs were able to quickly respond to nutritional status.

Retinoic acid directly regulated RALDH enzyme expression in LN stromal cells and dendritic cells.

Our data suggest that RA, derived from vitamin A, is crucial for RALDH expression in MLN-DCs and MLN stromal cells and we hypothesized that RA itself may directly induce RALDH expression in these cells. Indeed, several of the genes involved in the metabolic pathway of vitamin A are positively induced by RA [48-51], thus creating a positive feedback loop. We therefore determined whether RALDH expression in LN stromal cells and BM-DCs could be induced by addition of increasing amounts of RA *in vitro*. Expression of RALDH2 was significantly induced in PLN stromal cells by addition of 50 nM RA (Figure 5A, left), while at high RA concentrations

(500 nM), no induction of RALDH expression could be observed. Additionally, analysis of MLN stromal cells demonstrated a reduction of RALDH2 expression with increasing amounts of RA (Figure 5A, right). Furthermore, BM-DCs stimulated *in vitro* with 100 nM RA showed a 5-fold higher expression of RALDH2 mRNA, while induction of RALDH1 and RALDH3 enzymes was not significantly affected (Fig 5B). Since epithelial cells express RALDH1 enzymes and can thus produce RA [25-27,52], epithelial-derived RA might be an important imprinting factor for inducing RALDH2 expression in lamina propria-DCs. For MLN stromal cells the transport of vitamin A by the lymph may be of importance [53,54]. Overall, these data imply that RA can imprint both DCs and LN stromal cells in a dose dependent manner.

RALDH expression in small intestines is not affected by vitamin A deficiency.

Since RALDH expression in MLN-DCs and MLN stromal cells is dependent on vitamin A, we reasoned that the levels of RALDH expression in epithelial cells within the small intestine may also depend on dietary vitamin A. We tested small intestines from VAD and VAH mice for mRNA expression of RALDH1, RALDH2 and RALDH3 enzymes using real time PCR. Remarkably, during vitamin A deficiency, RALDH1 and RALDH2 mRNA expression levels were not altered and were comparable to levels observed in VAC small intestines (Figure 6A). RALDH3 expression was significantly increased during vitamin A deficiency, indicating the presence of a compensatory mechanism for RA production by increasing RALDH3 expression.

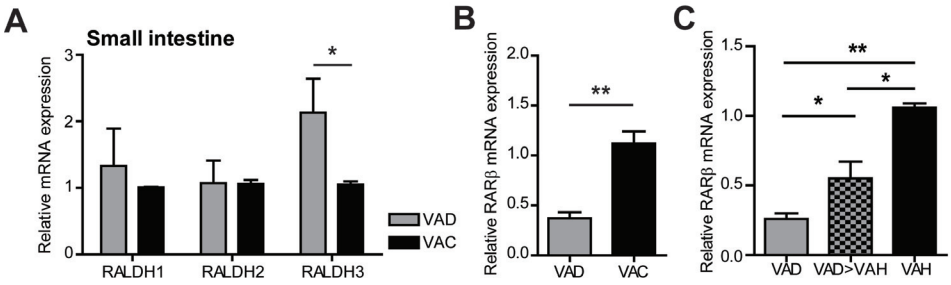

Figure 6. Expression of RALDH enzymes in small intestines is not affected by dietary vitamin A deficiency.

Expression of RALDH1, RALDH2, RALDH3 mRNA (A) and RAR□□mRNA (B) were analyzed in small intestine samples from VAD and VAC mice by real time PCR. Expression of transcripts was normalized to Cyclophilin A and Ubiquitin C. Relative expression levels in VAC small intestines was set at 1.0 for each gene analyzed. Per group 3 animals were used. Significant differences are indicated by * (p=0.02) and ** (p=0.001). (C) Expression levels of RAR□□mRNA were analyzed in small intestine samples from VAD, VAH and VAD mice supplemented with vitamin A (VAD>VAH) by real time PCR. Expression of transcripts was normalized to Cyclophilin A and Ubiquitin C. Relative expression levels in VAH small intestines was set at 1.0 for each gene analyzed. Per group 5 animals were used. Significant differences are indicated by * (p=0.02) and ** (p<0.001).

RAR□ is a nuclear receptor for RA and known to be a direct target gene of RA [48,50]. Expression levels of RARβ mRNA can thus be viewed as an indicator of RA-mediated signalling within cells. Analysis of RARβ showed that mRNA levels were indeed strongly reduced in VAD small intestine (Figure 6B), showing that RA-mediated signalling is absent within the intestine of VAD mice. The absence of RA-mediated signalling within the intestine could be restored by supplementation of VAD mice with dietary vitamin A for 7 days since RARβ expression was significantly induced in the supplemented VAD mice (VAD>VAH) when compared to VAD animals (Figure 6C). From this we can conclude that vitamin A supplementation of VAD mice leads to its conversion to RA and induction of RA-mediated signalling, resulting in the induction of RALDH expression MLN-DCs and MLN stromal cells.

Discussion

We have demonstrated that during postnatal development RALDH enzymes are upregulated in MLN-DCs and MLN stromal cells, pointing to environmental factors involved in the induction. RALDH activity in MLN-DCs was not affected when microbial triggering via TLRs was diminished, as seen in mice defective in Trif- and MyD88-dependent signalling, while the intake of vitamin A turned out to be instrumental for RALDH activity within the mucosal immune system. Not only RALDH expression in DCs, but also in MLN stromal cells was critically dependent on vitamin A consumption. Notably, RALDH enzyme activity in MLN-DCs could quickly be restored in vitamin A deficient animals by vitamin A supplementation. These data give further insight in the mechanisms of how vitamin A affects the functioning of the mucosal immune system.

Mice are essentially born germ-free, but soon after birth, mucosal surfaces are colonized with high numbers of bacteria [45,46]. Germ-free mice show reduced numbers of lamina propria $CD4^+$ T cells, IgA producing B cells and intra epithelial lymphocytes [55,56], and MLNs and spleens are smaller and less cellular [57]. This indicates that commensal bacteria play a crucial role in the maturation of the mucosal immune system during postnatal development. Therefore, we speculated that TLR triggering might be involved in the induction of RALDH expression, as suggested previously [37,42]. However, MLN-DCs from both $MyD88^{-/-}$ and Trif mutant animals had comparable levels of RALDH activity to WT animals *in vivo*. This suggests that the regulation of RALDH activity in MLN-DCs *in vivo* does not involve MyD88- and Trif-dependent TLR signalling, confirming the recent findings that MLN-DCs from mice deficient for both MyD88 and Trif still showed ALDELFUOR activity [47]. Nevertheless, it is conceivable that other pattern recognition receptor pathways may overcome the deficiency of Trif or MyD88 proteins *in vivo*. The literature indicates however that DCs, which are exposed to intestinal factors, express lower TLR levels and are thus less well able to respond

to TLR ligands [12,32,32,58,59]. This could explain our finding that MyD88 and Trif are dispensable for RALDH expression in CD103$^+$ MLN-DCs *in vivo*. In contrast, our data showed that vitamin A-derived RA is mandatory for RALDH expression and activity in MLN-DCs. Remarkably, in addition to the inhibitory effect of RA on TLR responsiveness in DCs, RA has been shown to increase intestinal epithelial barrier function [60,61]. Therefore, in case of vitamin A deficiency, the intestinal barrier might be compromised resulting in enhanced invasion of bacteria and enhanced exposure of DCs to TLR ligands. Despite potentially enhanced bacterial invasion and exposure, we could not observe RALDH enzyme activity in lamina propria-derived DCs during vitamin A deficiency.

Intestinal epithelial cells produce a vast array of soluble factors which are thought to license lamina propria DCs to induce gut-homing T cells through the expression of RALDH enzymes [34,35]. Factors produced by epithelial cells include RA, as well as GM-CSF, IL13, TGFβ, TSLP and IL-4. Of these factors, RA was shown to be crucial for the imprinting of the mucosal DCs phenotype (this paper and [35]). At first this seems to be contradicted by statements that RA could only weakly induce RALDH expression FLt3L derived BM-DCs [37], however the concentration used (1μM RA) was within the range at which we also fail to induce RALDH expression in BM-DCs. Both our data and data from others [35] showed that RA used at concentrations around 100 nM can significantly induce RALDH2 expression in BM-DCs while a further increase of RA seems to inhibit expression of RALDH2. Negative regulation of RALDH2 by RA has indeed been described to occur upon excessive RA administration during embryonic development [62]. Moreover, GM-CSF plus IL-4 have been described to contribute to the expression of RALDH enzymes in Flt3L-BM-DCs [37]. GM-CSF is produced in the intestines and MLNs by macrophages and granulocytes [37]. MLN-DCs from beta-c$^{-/-}$ animals, lacking the GM-CSF receptor, showed a reduction of approximately 50%, while the absence of dietary vitamin A resulted in a 94% reduction (Figure 3B). It is conceivable that GM-CSF within the small intestine is also controlled by RA and that VAD mice produce low levels of GM-CSF leading to lack of RALDH2 induction. We tested this hypothesis and found that, on the contrary, GM-CSF levels were not different in the small intestines and MLNs of VAD mice and VAC mice (Supplementary Figure 2). In addition to GM-CSF, IL-4 and TGF-β1 were tested and, although TGF-β1 was significantly lower in MLNs from VAD mice, levels of IL-4 and TGF-β1 were found not to be different in the small intestines of VAD mice and VAC mice (Supplementary Figure 2). We therefore conclude that lack of RALDH2 in MLN-DCs does not correlate with the absence of GM-CSF, IL-4 and TGF-β1 in VAD mice.

It is intriguing that RALDH levels in MLN DCs and stromal cells are slowly induced after birth. Vitamin A-derived RA is crucial for embryonic development and is transplacentally delivered from the mother to the embryo. Embryos, however, do not store this vitamin A and embryos and newborn mice have very low levels of vitamin A in their livers compared to adult mice [63,64]. Postnatally, newborn mice

receive high amounts of vitamin A via breast milk allowing to build up vitamin A stores in the liver. Perhaps that first sufficient liver stores need to be generated before vitamin A, bound to its transporter protein RBP4, can be transported through the blood in sufficient amounts to other tissues like intestines and MLNs. During vitamin A deficiency, RALDH expression could still be detected in the small intestines, while RARβ expression was virtually absent, indicating that RA-mediated signalling no longer occurred. Thus, although epithelial cells of VAD mice were still capable of converting vitamin A, lack of vitamin A resulted in the absence of RA and consequently in the failure to induce RALDH expression in lamina propria-derived DCs and MLN stromal cells. These data showed that dietary vitamin A has a profound effect on RALDH enzymes in DCs and stromal cells, while it does not regulate RALDH expression in the small intestine. Other factors, possibly present in the intestinal lumen, may contribute to RALDH expression by epithelial cells. Since intestinal epithelial cells express RALDH1 and have been shown to be able to imprint DCs in a RA-dependent manner [7,26,35], these cells are most likely the providers of RA for induction of RALDH in lamina propria DCs. It will therefore be of importance to establish whether RALDH levels in the intestinal epithelial cells are constant or whether they are also controlled by environmental factors.

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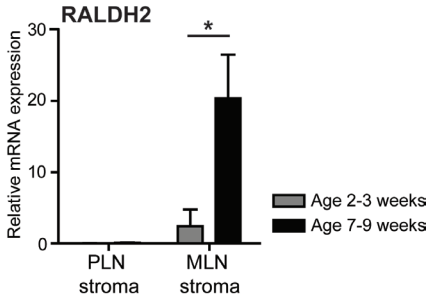
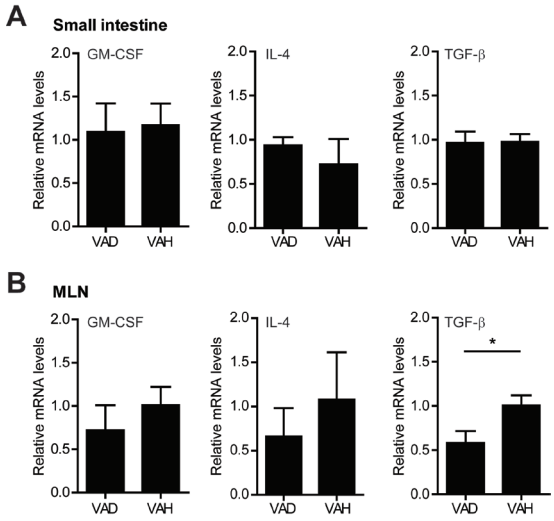
Supplemental data



Supplementary figure 1. RALDH expression in MLN stromal cells is increased during postnatal development.

RNA was isolated from FACS-sorted CD45$^-$ PLN and MLN stromal cells from young female C57BL/6 mice, aged 2-3 weeks, and from adult female C57BL/6 mice, aged 7-9 weeks. Cells were analyzed for relative expression levels of RALDH enzymes by real-time PCR. RALDH1 and RALDH3 expression were too low to give any consistent difference. Experiment was performed two times. Per group 3-6 animals were used for FACS-sorting. Significant difference is indicated by * ($p<0.001$).



Supplementary figure 2. GM-CSF levels in small intestines and MLNs remain unchanged during vitamin A deficiency.

Expression levels of GM-CSF, IL-4 and TGF-β1 mRNA were analyzed in small intestines (A) and MLNs (B) from VAD and VAH by real time PCR. Expression of transcripts was normalized Ubiquitin C. Relative expression levels in VAH small intestines (A) and VAH MLNs (B) was set at 1.0. In this experiment small intestines from 5 animals and MLNs from 3 animals were used for mRNA analysis. Significant difference is indicated by * ($p=0.003$).

5

Levels of vitamin A metabolism correlate with mucosal immune function

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5

Abstract

The vitamin A metabolite retinoic acid (RA) has been reported to suppress Th1 responses and enhance Th2 responses. Here we investigated whether differences in vitamin A metabolism could underlie the differences between C57BL/6 and BALB/c mice, which are reportedly seen as Th1 and Th2 responders, respectively. BALB/c mice were shown to have higher RALDH activity in MLN-DCs, increased ability to induce CCR9 expression on $CD4^+$ T cells and more FoxP3 regulatory T cells. Within BALB/c small intestines, higher expression levels of RALDH enzymes, RA-mediated signalling and IgA secretion was observed, as well as increased accumulation of T cells and B cells in the small intestinal lamina propria. Therefore, the level of RA production and consequently the degree of RA-mediated signalling is crucial for the efficiency of the mucosal immune system.

Introduction

Vitamin A has long been known for its role in immunity, especially since vitamin A deficiency ablates proper mucosal immune responses, leading to diarrhea, infections and early childhood mortality [1-3]. Vitamin A is a fat soluble vitamin and is absorbed from the gastrointestinal tract. It can be consumed preformed as retinyl esters, as found in animal source foods such as liver, egg, fish and whole fat dairy products [3]. Dietary vitamin A can also be obtained from provitamin A carotenoids as found in vegetables and fruits. Vitamin A is metabolized into the active metabolite retinoic acid (RA) in two oxidative steps. Vitamin A is first reversibly oxidized by alcohol dehydrogenases (ADH) to form retinaldehyde. Next, retinaldehyde is irreversibly metabolized to RA by three members of the aldehyde dehydrogenase gene family, ALDH1A1 (RALDH1), ALDH1A2 (RALDH2) and ALDH1A3 (RALDH3) [4-6]. The active metabolite RA binds to retinoic acid receptors (RARs) which in turn act as transcription factors that bind retinoic acid responsive elements within the promoter regions of target genes [7-9].

RALDH expression is associated with the mucosal immune system and reported to be functionally active within intestinal epithelial cells [10-12]. Also, dendritic cells (DCs) in Peyers' patches (PPs), mesenteric lymph nodes (MLNs) and intestinal lamina propria express RALDH enzymes, while splenic or peripheral LN (PLN)-DCs display only very low expression [13-17]. Furthermore, stromal cells within MLNs express RALDH enzymes [14,16]. We have shown that RA itself directly regulates the level of RALDH expression in BM-DCs as well as LNstromal cells *in vitro*, and that RALDH expression within the mucosal immune system requires dietary intake of vitamin A (chapter 4). Generation of RA is crucial for the induction gut-homing molecules $\alpha_4\beta_7$ and CCR9 on activated T cells [17]. Furthermore, the induction of FoxP3 regulatory T cells relies on RA, in combination with TGFβ [13,18-23], while it suppresses the differentiation of Th17 cells [18,21,22]. Moreover, RA is reported to skew immunoglobulin class switching in B cells to an increased secretory IgA production [24-26], which protects the host against the vast array of microbes constantly present in the intestinal lumen. Therefore, RA appears to be a key molecule that controls mucosal immune responses.

The genetic background of inbred mice was found to be a determing factor in the preferential induction of either a Th1 or Th2 immune response [27,28]. The subset of T cells termed Th1 cells, which produce interleukin-2 (IL-2), interferon-γ (IFNγ) and lymphotoxin, are important mostly for immunity against intracellular pathogens, such as *Leishmania major*. In contrast, Th2 cells produce IL-4, IL-5, IL-10 and IL-13, which contributes to the direct enhancement of humoral-mediated immunity. T cells from C57BL/6 mice preferentially produce Th1 cytokines with high IFNγ and low IL-4, whereas T cells from BALB/c mice favor Th2 cytokine production with low IFNγ and high IL-4 when stimulated *in vitro* [27]. Interestingly, it has been shown that RA can suppress Th1 responses and enhance

Th2 responses [15]. In this study we therefore investigated whether C57BL/6 and BALB/c mice differ in the metabolism of vitamin A and in mucosal immune functions that are RA-dependent. We demonstrate that MLN-DCs from BALB/c mice displayed higher RALDH activity, which correlated with increased induction of gut-homing molecules on $CD4^+$ T cells and more $FoxP3^+$ regulatory T cells. In addition, BALB/c mice showed higher expression levels of RALDH enzymes and RA-mediated signalling within the small intestines compared to C57BL/6 mice. Within the lamina propria of the small intestines, an increased accumulation of T cells and B cells was observed in BALB/c mice, with a concomitantly enhanced secretion of IgA into the intestinal lumen. In conclusion, enhanced RA production in BALB/c mice is associated with the development of a more efficient mucosal immune system.

Materials and Methods

Mice

C57BL/6 mice and BALB/c mice aged 10-14 weeks were obtained from Charles River (Charles River, Maastricht, The Netherlands). DO11.10 transgenic mice and OT-II transgenic mice were bred at the animal facility of the VU Medical center and used at the age of 7 weeks. Both DO11.10 mice, generated on a BALB/c background, and OT-II mice, generated on a C57BL/6 background, have CD4 T cells with a transgenic T cell receptor specific for the ovalbumin (OVA) 323-339 peptide. All mice were kept under standard animal housing conditions. The Animal Experiments Committee of the VU Medical Center approved all of the experiments described in this study.

T cell enrichment, CFSE labelling, transfer and antigenic stimulation

Spleens and LNs from DO11.10 and OT-II mice were minced through a 100-μm gauze to obtain single cell suspensions. To deplete erythrocytes from spleen cell suspensions, cells were incubated for 2 minutes on ice in lysis buffer (150 mM NH_4, 1 mM $NaHCO_3$, pH 7.4). $CD4^+$ cells were enriched using the CD4 negative selection kit (Dynal, Invitrogen, Breda, The Netherlands). Cells were labelled with 5 μM of 5,6-carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Invitrogen) at $3x10^7$ cells/ml for 10 min at 37° C, after which the cells were washed. BALB/c and C57BL/6 mice were injected with approximately $4x10^6$ OVA-specific T cells and received 50 mg OVA (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 200 μl saline 24 hours later by intragastric (i.g.) administration. Seventy-two hours after antigenic stimulation, MLNs were dissected and used as single cell suspensions for FACS analysis.

Flow cytometry and ALDEFLUOR assay

Single cell suspensions were made by cutting LNs with scissors, followed by digestion at 37ºC for 25 min while constantly stirring, using 0.5 mg/ml Blendzyme 2 (Roche, Penzberg, Germany) and 0.2 mg/ml DNAse I (Roche) in PBS. Cell clumps were removed by pipetting the cells over a nylon mesh. The LN cells were washed and resuspended in PBS with 2% NBCS.

RALDH activity in individual cells was measured using the ALDEFLUOR assay kit (StemCell Technologies, Grenoble, France), according to the manufacturer's protocol with modifications. Briefly, cells resuspended at 10^6 cells per ml in ALDEFLUOR assay buffer containing activated ALDEFLUOR substrate (365 nM) with or without the RALDH inhibitor diethylaminobenzaldehyde (DEAB, 7.5 µM) were incubated for 40 min at 37°C. For flow cytometric analysis of ALDEFLUOR-reacted cells, cells were subsequently stained with PE conjugated anti-CD11c (clone N418, eBioscience, Immunosource, Halle-Zoersel, Belgium), biotin conjugated anti-CD103 (clone M290, BD Bioscience, Breda, The Netherlands) Alexa fluor (Invitrogen, Breda, The Netherlands) 647 conjugated anti-MHC-II (clone M5/114) and with Sytox Blue (Invitrogen) to discriminate between live versus dead cells. Secondary antibody used was Percp conjugated (BD Bioscience) or PE-Cy7 conjugated streptavidin (eBioscience).

Upon OVA administration, MLNs were isolated from BALB/c and C57BL/6 mice and single cell suspensions were made by digestion as described above. Subsequently, cells were stained with biotin conjugated anti-mouse DO11.10 TCR (KJ1-26, Caltag Laboratories, Burlingame, CA) or PE conjugated anti-mouse Vα2 TCR (clone B20.1, eBioscience) to identify OVA-specific T cells in BALB/c and C57BL/6 mice, respectively. To analyze expression of gut-homing molecules, cells were also stained with anti-$\alpha_4\beta_7$integrin (clone DATK32, kindly provided by Dr. Alf Hamann, Charité Universitätsmedizin Berlin, Germany) and anti-CCR9 (clone 7E7, kindly provided by Prof. Dr. Reinhold Förster, Hannover Medical School, Hannover, Germany), in combination with PE-Cy7 conjugated anti-CD4 (clone GK1.5, eBioscience) and Sytox Blue (Invitrogen) to discriminate between live versus dead cells. Secondary antibody used was Alexa 647 conjugated anti-rat antibody (Invitrogen). Cells were analyzed with a Cyan ADP flow cytometer (Beckman Coulter, Mijdrecht, The Netherlands).

Intracellular FoxP3 staining

Cell suspensions were prepared from C57BL/6 and BALB/c MLNs by digestion as described above. Cells were stained with Alexa 488 conjugated anti-CD4 (clone GK1.5) and biotin conjugated anti-CD25 (clone 7D4, BD Bioscience). Secondary antibody used was PE-Cy7 conjugated streptavidin (eBioscience). For Foxp3 staining, cells were subsequently fixed in Fix/Perm buffer (eBioscience), followed by permeabilization and staining for Foxp3 with Alexa 647 conjugated anti-

Foxp3 (clone FJK-16s, eBioscience) according to the manufacturer's instructions (eBioscience). Cells were analyzed with a Cyan ADP flow cytometer (Beckman Coulter).

Enzyme-linked immunosorbent assay for secretory IgA

The faeces of the small intestines (SI) of C57BL/6 and BALB/c mice were collected in cold PBS buffer (faecal content of 33 cm SI in 2 ml PBS). Debris was removed by cold centrifugation for 20 min at 2000 rpm to harvest the supernatant for analysis of secretory IgA after an appropriate dilution. Secretory IgA was determined by sandwich-ELISA according to the manufacturer's protocol (Gentaur Europe, Brussels, Belgium), in which the anti-mouse IgA capture antibody was coated on the plate and bound faecal IgA was detected with horseradish peroxidase labeled anti-mouse IgA detecting antibody. Samples were analyzed with a Fluostar Optima microplate reader (BMG Labtech, Isogen Lifescience, de Meern, The Netherlands). The concentration of secretory IgA was expressed as µg/ml PBS-dissolved faeces.

RNA isolation and real time PCR

Small intestines were dissected from C57BL/6 and BALB/c mice, flushed with PBS and homogenized in TRIZOL (Gibco, Invitrogen). RNA was isolated by precipitation with isopropanol. The concentration of RNA was measured with the Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA was synthesized from total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Burlington, Canada) according to the manufacturer's protocol.

Mesenteric lymph node cells from C57BL/6 and BALB/c were lysed and mRNA was isolated using the mRNA capture kit (Roche). cDNA was synthesized with the Reverse Transcriptase kit (Promega, Leiden, The Netherlands) according to the manufacturer's protocol.

Specific primers for CD45, CD3, CD19, Foxp3, *Csf2* (GM-CSF), IL-4, IFNγ, RARβ, RALDH1 (*Aldh1A1*), RALDH2 (*Aldh1A2*), and RALDH3 (*Aldh1A3*) and primers for housekeeping genes Ubiquitin C and GAPDH (Isogen Life Science, De Meern, Netherlands; Invitrogen) were designed across exon-intron boundaries using Primer Express software (PE Applied Biosystems, Foster City, CA) and Vector NTI software (Invitrogen). Real time PCR analysis was performed on an ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems). Total volume of the reaction mixture was 10 µl, containing cDNA, 300 nM of each primer and SYBR Green Mastermix (PE Applied Biosystems).

Statistics

Results are given as the mean ± SD. Statistical analyses were perfomed using the 2-tailed Student's *t* test.

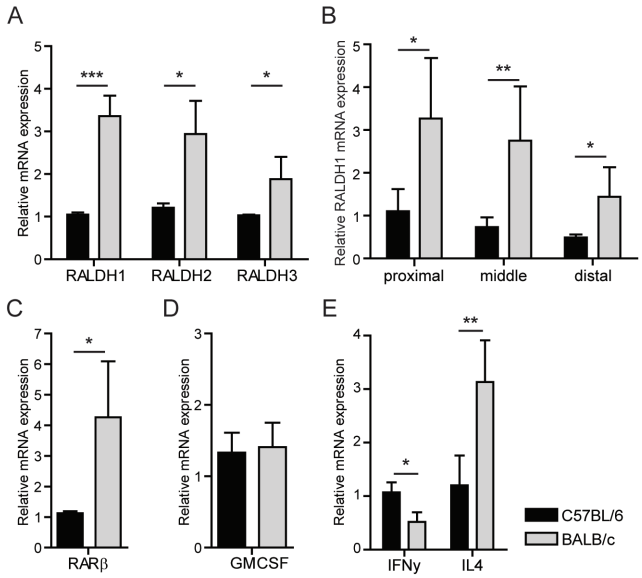

Figure 1. RALDH enzyme expression is increased in BALB/c small intestine.

Expression levels of RALDH1, RALDH2, RALDH3 mRNA (A), RARβ mRNA (C) and GM-CSF (D) were analyzed in small intestine samples from C57BL/6 (black bars) and BALB/c (grey bars) by real time PCR. Expression of transcripts was normalized to Ubiquitin C. Relative expression levels in C57BL/6 small intestines was set at 1.0 for each gene analyzed. Per group 5 animals were used. (B) Expression of RALDH1 mRNA was analyzed in proximal, middle and distal parts of the small intestine. Expression of transcripts was normalized to Ubiquitin C. Relative expression levels in the proximal C57BL/6 small intestine was set at 1.0. Five animals were used for analysis. (E) Expression levels of IFNγ and IL-4 mRNA were analyzed in whole small intestine samples from C57BL/6 and BALB/c by real time PCR. Expression of transcripts was normalized to CD3 mRNA levels. Relative expression levels in C57BL/6 small intestines was set at 1.0 for both genes analyzed. Per group 5 animals were used. Significant differences are indicated by * ($p<0.05$), ** ($p=0.02$), or *** ($p=0.001$).

Results

BALB/c express higher levels of RALDH enzymes in small intestines compared to C57BL/6.

Since BALB/c mice are known as prototypical Th2-type mice and RA has been described to skew T cells towards the Th2-type profile upon activation, while inhibiting Th1 cytokine production [15,18], we hypothesized that the expression of RA-producing enzymes may be differentially expressed by BALB/c and C57BL/6 mice. Since epithelial cells from the small intestines are known to express RALDH enzymes [10-12], we decided to compare RALDH mRNA levels in the small intestines of both mouse strains with real time PCR. Indeed, expression of RALDH1, -2, and -3 was significantly enhanced in BALB/c small intestines compared to C57BL/6 (Figure 1A). The largest difference was observed for RALDH1, which is known to be highly expressed by epithelial cells lining the gut [17,29]. Remarkably,

when expression of RALDH1 was analyzed along the gut axis, starting at the stomach, we observed the highest expression in the proximal small intestine, while expression decreased towards the distal part of the small intestines (Figure 1B). In all three parts analyzed, RALDH1 expression was significantly higher in BALB/c mice compared to C57BL/6 mice.

RARβ is a retinoic acid receptor, known to be a direct target gene of RA [30,31]. Expression levels of RARβ mRNA can therefore be viewed as an indicator of RA-mediated signalling in cells. Analysis of RARβ showed that mRNA levels were significantly increased in BALB/c small intestine compared to C57BL/6, indicating that within the small intestines of BALB/c mice more RA-mediated signalling is taking place (Figure 1C). Since a relation between GM-CSF-mediated signalling and RALDH expression in DCs has been suggested in literature [32] we also checked whether GM-CSF levels were different between the 2 mouse strains. No significant difference could be found (Figure 1D). As a control, we analyzed IFNγ and IL-4 mRNA levels, corrected for CD3 mRNA expression, in small intestines as markers for Th1- and Th2-skewing, respectively. As expected, IFNγ expression was significantly higher in C57BL/6 small intestines, while the expression of IL-4 mRNA was significantly higher in small intestine samples from BALB/c mice (Figure 1E).

BALB/c MLN-DCs show higher RALDH activity than C57BL/6 MLN-DCs.

Studies have shown that CD103$^+$ MLN-DCs represent a population of migratory DCs derived from the lamina propria, transporting orally derived Ag from the intestine to the MLN and inducing gut-tropic T cells and regulatory T cells [33,34]. It has been hypothesized that DC acquire the mucosal phenotype within the intestinal environment, which is created by the epithelial cells. Indeed, we and others have shown that contact of BM-derived DCs with gut epithelial cells induced expression of RALDH enzymes and educated BM-derived DCs to induce gut-homing T cells *in vitro* [35,36]. Furthermore, we have shown that RA directly induces RALDH enzyme expression levels in BM-DCs *in vitro* (chapter 4) and that in the absence of RA, RALDH expression is lacking in CD103$^+$ DCs in MLNs. We therefore tested whether the increased RA-mediated signalling observed in small intestines of BALB/c mice could result in higher RALDH enzyme activity in CD103$^+$ MLN-DCs when compared to C57BL/6 mice. RALDH activity was measured using the ALDEFLUOR assay, a flow cytometry-based assay with a fluorescent substrate which is specific for RALDH enzymes. Notably, the percentage of MHCII$^+$ CD11c$^+$ DCs was decreased (Fig. 2A, left), but the proportion of DCs expressing CD103 was higher in MLNs from BALB/c compared to C57BL/6 mice (Figure 2A, right). Flow cytometry plots of ALDEFLUOR and CD103 showed that most of the ALDEFLUOR signal could be observed in the CD103$^+$ DC population of MLNs in both BALB/c and C57BL/6 mice (Figure 2B), confirming the report that CD103$^+$ MLN-DCs express higher RALDH mRNA levels compared to CD103$^-$ DCs in MLNs [13]. In addition, calculations of

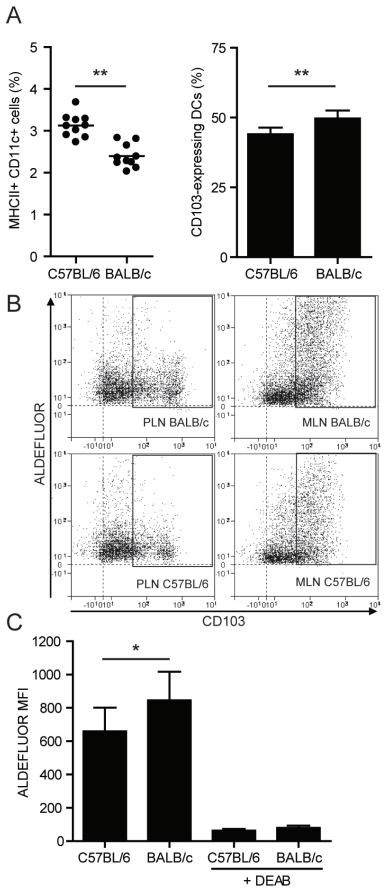

Figure 2. RALDH activity is increased in MLN-DCs from BALB/c mice.

(A-C) MHC-II$^+$ CD11c^{high} DCs in MLN cell suspensions from C57BL/6 and BALB/c mice were analyzed for CD103 expression and RALDH activity using the ALDEFLUOR assay. Data represent percentage MHC-II$^+$ CD11c^{high} DCs of total LN cells in individual MLNs (A, left) and average percentage ± SD of MHC-II$^+$ CD11c^{high} DCs expressing CD103 (A, right). (B) Representative FACS plots are shown for ALDEFLUOR signal and CD103 expression by MHC-II$^+$ CD11c^{high} DCs in PLN and MLN. Box indicates CD103$^+$ gate for calculations of ALDEFLUOR MFI in C. (C) Graph shows average ALDEFLUOR MFI ± SD in CD103$^+$ MHC-II$^+$ CD11c^{high} MLN-DCs in absence or presence of RALDH inhibitor DEAB. Per group a total of 10 animals were used for analysis in two separate experiments. Significant differences are indicated by * (p=0.03), ** (p<0.001).

ALDEFLUOR MFI revealed that RALDH activity was significantly higher in BALB/c CD103$^+$ MLN-DCs compared to C57BL/6. This activity was RALDH specific, since ALDEFLUOR MFI was highly decreased in presence of RALDH inhibitor DEAB and no difference between BALB/c and C57BL/6 could be observed (Figure 2C).

Induction of gut-homing T cells is enhanced in BALB/c MLNs.

Due to their highest RALDH2 expression, CD103$^+$ MLN-DCs are now being recognized as the DC subset that is best equipped to induce RA-mediated signalling in T cells, leading to FoxP3 T cell differentiation, and the induction of gut homing molecules [13,37,38]. Since BALB/c mice displayed higher RALDH activity in MLN-DCs when compared to C57BL/6, we addressed whether the induction of gut-homing T cells was different in BALB/c mice when compared to C57BL/6 mice. Hereto, CFSE-labelled ovalbumin (OVA) specific transgenic CD4$^+$ Vα2$^+$ T cells (OT-II cells) and OVA-specific CD4$^+$ KJ1$^+$ T cells (DO.11.10 T cells) were transferred to C57BL/6 and BALB/c mice, respectively. Twenty four hours later, mice received 50 mg ovalbumin intragastrically and at 72 hours after antigen

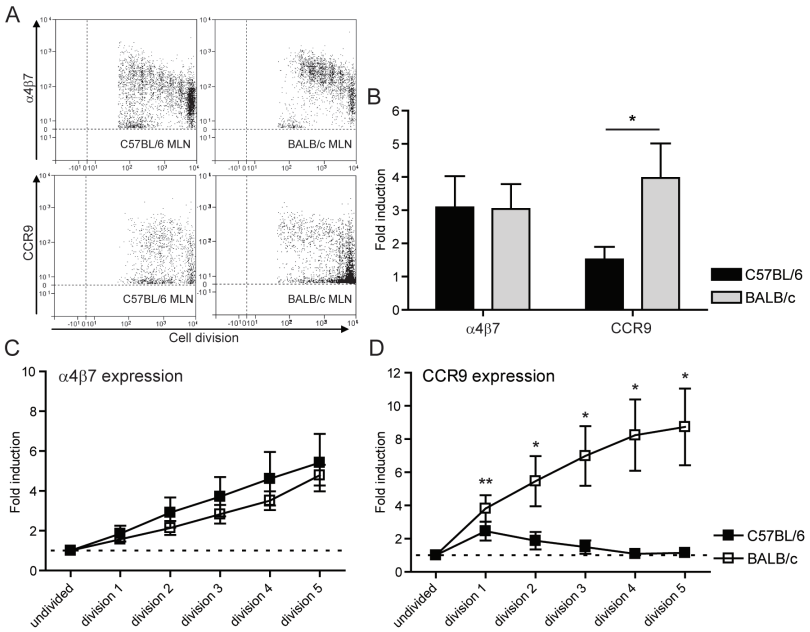

Figure 3. Induction of gut-homing molecules is enhanced in BALB/c MLNs.

(A-D) C57BL/6 and BALB/c mice received 4x10^6 OVA-specific CD4$^+$ OT-II or CD4$^+$ DO11.10 T cells respectively. Activated OT-II cells and DO11.10 cells in MLNs were analyzed by flow cytometry 3 days after intragastric OVA administration. Shown are flow cytometry plots of cell proliferation and expression of a_4b_7 (A, top) and CCR9 (A, bottom) by proliferating OT-II cells in C57BL/6 MLNs and proliferating DO11.10 cells in BALB/c MLNs. (B) Data represent average fold induction ± SD of $\alpha_4\beta_7$ and CCR9 expression on activated OVA-specific T cells compared to undivided T cells in C57BL/6 MLNs (black bars) and BALB/c MLNs (grey bars). (C, D) Data are calculated as average fold induction ± SD of a_4b_7 expression (C) and CCR9 expression (D) by activated OVA-specific T cells per cell division in MLNs from C57BL/6 (black squares) and BALB/c (white squares). Per group a total of 10 animals were used for analysis in two separate experiments. Significant differences are indicated by * ($p<0.003$), ** ($p=0.02$).

administration, expression of mucosal homing molecules $\alpha_4\beta_7$ and CCR9 was analyzed on proliferating OT-II and DO.11.10 T cells in MLNs by flow cytometry. No difference in $\alpha_4\beta_7$ and CCR9 expression was visible on the CFSE-labeled non-proliferating T cell populations in MLNs from both mouse strains, indicative of a uniform entry of injected cells in both BALB/c MLNs and C57BL/6 MLNs (Figure 3A). Flow cytometry plots also showed that in both BALB/c MLNs and C57BL/6 MLNs T cell activation is necessary for induction of gut-homing molecules $\alpha_4\beta_7$ and CCR9 (Fig. 3A). We observed that the fold induction of $\alpha_4\beta_7$ expression by proliferating OVA-specific T cells in C57BL/6 MLNs is similar to the fold induction in BALB/c MLNs (Figure 3B, left). Moreover, the fold induction of this gut-homing molecule analyzed per cell division showed a similar pattern in both mouse strains (Figure 3C, left). Surprisingly, the fold induction of CCR9 expression by proliferating OVA-specific T cells in BALB/c MLNs was much higher when compared to C57BL/6

MLNs (Figure 3B, right). Examining the fold induction of CCR9 expression per cell division revealed that CCR9 was induced on OVA-specific T cells in C57BL/6 mice during the first cell divisions, but then reached levels similar to the undivided peak (Figure 3C). Lack of a significant induction of CCR9 is probably caused by the generation of both $CCR9^-$ and $CCR9^+$ OVA-specific T cells in later cell divisions, which decreased the MFI of CCR9 expression of the entire dividing population (Figure 3A, left bottom). In BALB/c MLNs, however, CCR9 expression levels continued to increase with every cell division (Figure 3D). Unfortunately, we could not compare the induction of gut-homing molecules on $CD8^+$ T cells, because no transgenic mouse strain that recognizes OVA in the context of MHC-I on the BALB/c background exists. In conclusion, the results strongly suggest that higher RALDH acitivity in BALB/c mice correlated with a more efficient induction of gut-homing molecule CCR9 on T cells.

BALB/c mice display higher FoxP3 expression than C57BL/6 mice.

In addition to the essential role of RA in the induction of gut-homing molecules on T cells, RA also has been reported to be involved in the induction of FoxP3 expressing $CD4^+$ regulatory T cells [13,18-23]. We therefore tested FoxP3 mRNA levels in small intestine and MLN samples of both C57BL/6 and BALB/c mice with real time PCR. FoxP3 mRNA levels were significantly enhanced in both BALB/c MLN (Figure 4A) and BALB/c small intestines (Figure 4B) compared to the same tissues derived from C57BL/6. Moreover, FACS analysis of intracellular FoxP3 expression by T cells showed that the percentage of $CD25^+$ $FoxP3^+$ T cells was significantly higher in MLNs from BALB/c mice compared to C57BL/6 mice (Figure 4C). In conclusion, higher RALDH activity in BALB/c mice correlated with higher percentages of $FoxP3^+$ T cells present in MLNs.

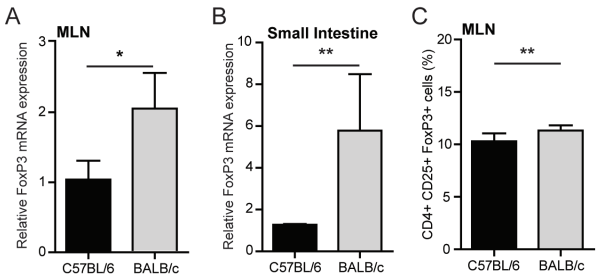

Figure 4. FoxP3 expression is increased in BALB/c small intestines and MLNs.

(A, B) Expression of FoxP3 mRNA was analyzed in MLN (A) and small intestine (B) samples from C57BL/6 (black bars) and BALB/c mice (grey bars) by real time PCR. Expression of transcripts was normalized to Ubiquitin C for small intestine and to GAPDH and Ubiquitin C for MLN. Relative expression levels in small intestines and MLNs from C57BL/6 mice was set at 1.0. Per group 5 animals were used. (C) Single cell suspensions of MLNs from C57BL/6 (black bars) and BALB/c mice (grey bars) were stained for CD4 and Foxp3 and analyzed by FACS. Data represent average percentage ± SD of $CD25^+$ $FoxP3^+$ among $CD4^+$ T cells. Per group 6 animals were used. Significant differences are indicated by * ($p<0.003$), ** ($p<0.05$).

BALB/c mice display increased lymphocyte levels and IgA secretion in small intestines.

Since the induction of gut-homing T cells occurred with greater efficiency in BALB/c MLNs when compared to C57BL/6 MLNs, we questioned whether this would result in increased numbers of immune cells in the small intestinal lamina propria. With immunostainings we observed more hematopoietic cells in the lamina propria of small intestines of BALB/c mice when compared to C57BL/6 (data not shown). To quantify this, expression levels of CD45 (haematopoietic cells), CD3 (marker for T cells) and CD19 (marker for B cells) were analyzed in the small intestines from C57BL/6 and BALB/c mice with real time PCR. Data showed that the expression of CD45 mRNA in BALB/c small intestine was increased about 2-fold compared to C57BL/6 (Figure 4A). In addition, mRNA levels for CD3 and CD19 were significantly higher in BALB/c small intestines when compared to C57BL/6 small intestines.
Since RA has been demonstrated to promote class switching of immunoglobulins to the IgA isotype in B cells [24-26], we wondered whether the secretion IgA into the intestinal lumen would differ between BALB/c and C57BL/6 mice. Faeces was collected from the small intestines of BALB/c and C57BL6 mice in which secretory IgA was measured by ELISA. We observed that the concentration of IgA within the faeces of BALB/c mice was remarkably higher compared to C57BL/6 mice (p=2.96E-10, Figure 5B). This could be the result of increased RA-mediated signalling within BALB/c small intestines as well as of increased numbers of B cells present in the small intestines. In conclusion, enhanced RA-mediated signalling in BALB/c mice correlated with increased induction of gut-homing molecules, with enhanced migration of lymphocytes to the small intestines, as well as with elevated levels of secretory IgA within the intestinal lumen.

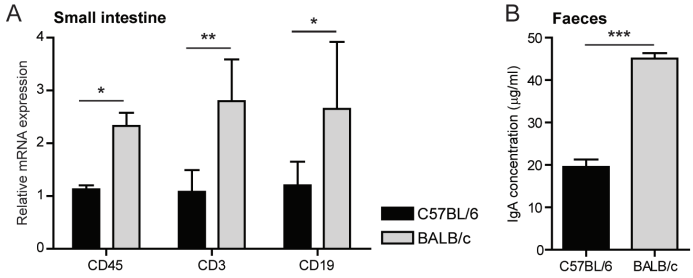

Figure 5. Increased lymphocyte levels and IgA secretion in BALB/c small intestines.
(A) Expression of CD45, CD3, and CD19 mRNA was analyzed in whole small intestine from C57BL/6 and BALB/c by real time PCR. Expression of transcripts was normalized to Ubiquitin C. Per group 5 animals were used. Relative expression levels in C57BL/6 small intestines was set at 1.0 for each gene analyzed. (B) Concentration of secretory IgA (mg/ml of PBS-dissolved faeces) in faeces from C57BL/6 mice (black bars) and BALB/c mice (grey bars) measured by ELISA. Per group 6 animals were used. Significant differences are indicated by * ($p<0.05$), ** ($p=0.003$), *** ($p=2.96E-10$).

Discussion

In this study we demonstrated that C57BL/6 and BALB/c mice differ in the expression and activity of RALDH enzymes and consequently in RA-induced immune functions which are crucial for the mucosal immune system. BALB/c mice displayed enhanced vitamin A metabolism, since expression levels of RALDH enzymes and RA-mediated signalling in small intestines were increased, as well as RALDH activity in $CD103^+$ MLN-DCs. Consequently, increased induction of CCR9 expression on $CD4^+$ T cells was observed, suggesting that activated T cells from BALB/c MLNs are better equipped to migrate to the intestines. Indeed, we observed increased mRNA levels for T cells and B cells in the small intestines of these mice. In addition, higher levels of secretory IgA were found in the intestinal lumen of BALB/c mice when compared to C57BL/6 mice. Moreover, FoxP3 mRNA was increased in small intestines and MLNs of BALB/c mice. Although we observed a difference in percentage of $FoxP3^+$ T cells in MLNs from BALB/c versus B6 mice, we did not discriminate between naturally occurring regulatory T cells, which are thymus derived, and regulatory T cells that differentiated in the periphery towards FoxP3 expressing T cells. Whether there is a role for RA in the induction of regulatory T cells in the thymus is not clear.

Since RA-mediated signalling is crucial in shaping the mucosal immune system, it becomes important what the underlying cause of the observed differences between BALB/c and C57BL/6 is. Notably, it has been published that GM-CSF is an important factor involved in imprinting of RALDH expression in mucosal DCs [32]. However, in contrast to the difference in RALDH levels, we observed no significant difference in GM-CSF mRNA levels in BALB/c and C57BL/6 small intestines (Figure 1D). We therefore propose that GM-CSF is not directly involved in imprinting of intestinal DCs, but that it may contribute at another level to RALDH expression. What other factors are responsible for the difference in RA production and signalling in these mouse strains remains unknown. Cellular RA availability is regulated by the vitamin A nutritional status and the tightly regulated balance between RA synthesis and catabolism. Notably, BALB/c mice and C57BL/6 mice used in these studies were kept under similar housing conditions and nutritional status. Possibly, factors that regulate the balance between RA synthesis and catabolism are differentially expressed in BALB/c and C57BL/6. Polymorphisms and mutations have been reported to exist in some genes that encode these factors [39-46]. Future research is needed to establish whether these mutations or polymorphisms indeed affect the levels of RA synthesis.

We propose that enhanced RA-mediated signalling seen in BALB/c mice when compared to C57BL/6 mice may lead to a more efficient mucosal immune system. Consequently, BALB/c may combat mucosa-associated pathology better. In fact, it has been shown that BALB/c mice are more resistant to developing colitis and require a higher dose of DSS compared to C57BL/6 to induce a comparable disease severity [47-49]. Even with the higher dose of DSS, BALB/c mice lose less

weight and recover more rapidly after withdrawal of DSS compared to C57BL/6 mice. These differences are often attributed to a difference in Th1-Th2 balance in these mice, since BALB/c mice are known as prototypical Th2-type mice, while C57BL/6 mice show a more Th1-driven response. In addition, treatment with RA ameliorated human and murine colitis by increasing the number of Treg cells [50]. We propose here that variation in RA metabolism might determine disease susceptibility in these mice, since RA affects homing of T cells to the intestines as well as the generation of FoxP3$^+$ regulatory T cells and isotype switching toward IgA. Moreover, RA availability determines epithelial integrity and increases the barrier function of the intestinal epithelial cells [51,52].

Although BALB/c mice are resistant to developing DSS-induced colitis, these mice are more susceptible when compared to C57BL/6 to infections that require a typical Th1 type response, such as *Leishmania major* infection in the skin. Resistant C57BL/6 T lymphocytes produce IFNγ that activates macrophages to produce NO and kill the parasite, while susceptible BALB/c T lymphocytes instead produce more IL-4 that suppresses macrophages [53,54]. Therefore, BALB/c mice show enhanced mucosal immune system at the expense of the peripheral immune system.

An example of a typical Th17-driven immune disease is experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis (MS) [55]. Strain specific differences have been reported to exist in C57BL/6 and BALB/c mice during EAE [56,57]. Disease onset in BALB/c mice was delayed when compared to C57BL/6 mice. Clinical signs of EAE rapidly decreased in BALB/c mice while C57BL/6 mice showed a slow decline of disease severity [56]. Given the fact that RA has been described to skew T cells upon their activation towards the Th2-type profile, while inhibiting Th1 and Th17 cytokine production [15,18], a number of research groups have investigated the effect of RA treatment on EAE disease progression and severity. RA was shown to strongly inhibit pathogenic Th17 and Th1 responses during EAE. Treatment with RA dramatically delayed disease onset and strongly decreased disease severity [58-61]. Other Th17-driven infectious diseases might therefore also benefit from RA treatment as was reported for the murine model for rheumatoid arthritis [62].

Since enhanced RA-mediated signalling may lead to a decreased Th1-Th2 balance, increased induction of Foxp3$^+$ regulatory T cells and epithelial barrier function, modulation of RA availability would be beneficial to increase the mucosal immune response and resistance to infections and immune diseases. Studies in vitamin A–deficient patients showed indeed a compromised Th2-type immune response and a cytokine imbalance skewed towards Th1 [63].

In conclusion, our data suggest that the enhanced RA synthesis in BALB/c mice when compared to C57BL/6 mice results in the development of a more efficient mucosal immune system. Control of RALDH levels may therefore be an attractive way to regulate the mucosal immune response.

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6

Levels of vitamin A metabolism correlate with colitis severity

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6

Abstract

Dextran sulphate sodium (DSS)-induced colitis is a widely used animal model for inflammatory bowel diseases. The BALB/c inbred strain of mice is more resistant to DSS-induced colitis than C57BL/6 mice; however, the mechanism underlying this phenomenon is largely unknown. In this paper, we show that BALB/c mice have more organized gut associated lymphoid tissue (GALT) in the colon compared to C57BL/6 mice. Furthermore, decreased susceptibility to colitis is paralleled by increased colonic expression of retinaldehyde dehydrogenase 1 (RALDH1) in BALB/c mice. RALDH1 is an enzyme responsible for the conversion of vitamin A to its active metabolite retinoic acid (RA), which is involved in the induction of FoxP3^{+} regulatory T cells and IgA producing B cells. As we show here, removal of vitamin A from the diet of C57BL/6 mice, which will decrease RA levels, gave rise to a more severe form of DSS-induced colitis. This illustrates the importance of vitamin A for the control of the disease. Higher levels of RA-mediated signalling in the colon may thus serve as an explanation as to why BALB/c mice are resistant to DSS colitis and highlights the role of vitamin A and its metabolite in inflammatory bowel disease.

Introduction

Ulcerative colitis and Crohn's disease, collectively referred to as inflammatory bowel disease (IBD), are characterized by chronic inflammation of the intestinal tract. A variety of animals models exists for IBD. Dextran sulphate sodium (DSS) is a chemical compound, which is thought to have toxic effects on the epithelial cells lining the intestines, resulting in altered gut permeability and superficial intestinal inflammation that resembles, to a certain extent, ulcerative colitis [1, 2]. It has been reported by various independent research groups that strain specific differences exist in susceptibility to DSS-induced colitis between C57BL/6 and BALB/c inbred strains of mice [3-5]. In short, it has been shown that BALB/c mice are more resistant to developing colitis and require a higher dose of DSS compared to C57BL/6 to induce a comparable severity of disease. Even with higher doses of DSS, BALB/c mice generally lose less weight and recover more quickly after withdrawal of DSS as than C57BL/6 mice.

Vitamin A (retinol) is a fat soluble vitamin which has a variety of effects on the immune system. Vitamin A is absorbed from the gastrointestinal tract and converted to its active form retinoic acid (RA) through two oxidative steps. Retinol is converted to retinaldehyde by alcohol dehyrogenase enzymes. Retinaldehyde is subsequently oxidized by retinaldehyde dehyrogenases (RALDH) to form RA, which is an irreversible process [6-12]. RA then binds to retinoic acid receptors (RARs) which act as transcription factors and bind retinoic acid responsive elements (RAREs) within the promoter region of their target gene, leading to a variety of downstream effects [13, 14].

Retinoic acid has numerous effects on the immune system. Iwata and colleagues have shown that RA can suppress Th1 response and enhance Th2 responses [15]. Furthermore, RA is described to have an effect on lymphocyte homing to the intestine by inducing expression of the gut homing molecules $\alpha 4\beta 7$ and CCR9 on activated T cells [16, 17]. In combination with TGFβ, RA has also been shown to greatly enhance the differentiation, expansion and gut homing of T regulatory cells which serve to suppress immune responses [18-20]. RA not only affects T cells in the intestines but has also been reported to have numerous effects on B cells in the gut. RA aids in isotype class switching of immunoglobulins by B cells in gut-associated lymphoid tissue (GALT) leading to an increase in IgA production [21]. IgA is the most abundant immunoglobulin present in the intestines and secretory IgA forms a protective layer which lines the intestines and protects the host against the vast array of microflora constantly present in the intestines [22, 23]. RA has also been shown to induce the expression of CXCL13 during lymph node development and upon vagus nerve stimulation in the intestine [24]. Upregulation of CXCL13 in the intestine could lead to the attraction of more B cells to the intestine [25]. Thus RA has numerous beneficial effects on the mucosal immune system and an increase in RA-mediated signalling may aid the host in recovering from colitis.

Interestingly, it has been shown that BALB/c mice tend to have a more Th2 skewed cytokine profile whereas C57BL/6 mice tend to have a more Th1 skewed cytokine profile [26-30]. Taken together with the data showing that RA can suppress Th1 responses and enhance Th2 responses, this suggests that differences in RA production between these mouse strains may be responsible for the differences observed in colitis susceptibility. In this study we aimed to investigate this hypothesis by relating the expression levels of RALDH and RA-mediated signalling in the colon of C57BL/6 mice versus BALB/c to differences in DSS-induced colitis susceptibility.

Materials and Methods

Mice

Specific pathogen free (SPF) BALB/c, C57BL/6 female mice and C57BL/6 breeding pairs were obtained from Charles River (Charles River Laboratories, Maastricht, The Netherlands) and housed in the animal facility at the Vrije Universiteit (Amsterdam, The Netherlands). Animals were housed under standard laboratory conditions, only female mice were used in experiments. The starting age of the animals was 8-10 weeks with a weight range of 18-20 grams.
Generation of vitamin A deficient and vitamin A control animals was based on a method previously described by Iwata *et al* [17]. In short, females were mated and pregnant females were switched to the relevant vitamin A diet at 7-10 days of gestation and kept on the relevant diet until the pups were weaned. The offspring was subsequently placed on the same diet throughout the duration of the experiment. Two different custom made diets were based on modified AIN-93M diet (MP Biomedicals United States, Solon OH, United States): (1) vitamin A deficient diet containing no vitamin A and (2) vitamin A control diet containing 2800 IU/kg vitamin A. Ethics committee approval for all animal experiments was obtained from the Vrije Universiteit Animal Ethics Committee.

Induction of DSS colitis

We made use of a previously described single dose DSS colitis model by Melgar *et al* [3]. As previously established by other research groups BALB/c mice are more resistant to DSS-induced colitis than C57BL/6 mice [3-5]. Thus in order to induce a comparable severity of disease in both mouse strains we administered 2% DSS in C57BL/6 mice and 5% in BALB/c mice. A DSS titration (2%, 3%, 4% and 5%) was performed in BALB/c mice to confirm that in our facility 5% DSS in drinking water did indeed give a comparable severity of disease in BALB/c when compared to C57BL/6 mice given 2% DSS in drinking water, as reported (Melgar et al). C57BL/6

mice were thus given 2% DSS and BALB/c mice were given 5% DSS in drinking water *ad libitum* which was changed on a daily basis. For the acute colitis time point, analyzed at day 7, mice were given DSS for 7 days prior to euthanasia. For the chronic colitis time point mice were given DSS for the first 5 days of the experiment and then changed to normal drinking water for a 30 day rest period and euthanized at day 35.

Immunofluorescence

Mice were euthanized, colons were removed and embedded in OCT compound (Sakura Finetek Europe) and stored at -80°C. Tissues were subsequently sectioned on the cryostat (7 microns), and either stained with hematoxylin and eosin (H&E staining) or acetone fixed after which immunofluorescence stainings were performed. For an overview, pictures of a representative length of colon were visualized making use of the stitch picture function of the DM6000 Leica Immunofluorescence Microscope Leica (Leica Microsystems) with a 20x objective. For higher magnification pictures use was made of the Leica TCS-SP2-AOBS Confocal Laser Scaninning Microscope (Leica Microsystems).

Antibodies

Anti-gp38 (clone 8.1.1, anti-podoplanin) and anti-CD3 (clone KT3) were used as supernatants and visualized by means of the appropriate secondary antibody labeled with Alexa-Flour 488, Alexa-Flour 546 or Alexa-Flour 647 (Invitrogen Life Technologies, Breda, The Netherlands). Anti-B220 (clone 6B2) antibodies were affinity purified from hybridoma cell culture supernatants with protein G-Sepharose (Pharmacia, Uppsala, Sweden) and labeled with Alexa-Fluor 488 or Alexa-Fluor 647 (Invitrogen, Breda, The Netherlands). Anti-CXCL13-bio (R&D Systems, Minnneapolis, USA) was visualized using the TSA signal amplification Kit with HRP-streptavidin and Alexa Fluor 546 tyramide (Invitrogen). Anti IgA-bio (Invitrogen, Breda, The Netherlands) was used in combination with Alexa-Fluor-647 conjugated streptavidin (Invitrogen, Breda, The Netherlands) and Alexa-647 conjugated anti-FoxP3 was used (eBioscience, San Diego, CA, USA).

Real time PCR

Whole colons were removed and placed in TRIZOL (Gibco, Invitrogen Lifes Sciences,Breda, The Netherlands) and stored at -80^0 C. Entire colons were homogenized and RNA was isolated according to the manufacturer's instruction. The concentration of RNA was assessed by means of the Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA was synthesized by making use of a reverse transcriptase reaction which was performed according to the MBI Fermentas cDNA synthesis kit (Vilnius, Lithuania), using both the oligo(dT)18 and the D(N)6 primers.

Quantitative real time PCR was performed on the ABI Prism 7900 Sequence Detection System (Applied Biosystems , Foster City, CA). The reactions were performed with 0.25ng cDNA in a total volume of 10 μl containing SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 300nM of each primer according to the manufacturer's instructions. Primers were designed using Primer Express Software (Applied Biosystems) according to the guidelines provided by the manufacturer. Data obtained by real-time PCR were normalized for the geometric mean of the two most stable house-keeping genes (cyclophilin, ubiquitin) as determined by analysis with geNorm method software (http://medgen.ugent.be/~jvdesomp/genorm/).

Statistics

Data obtained for percentage of daily weight loss and/or weight gain was analyzed by means of repeated measurement statistics to assess effect of day and effect of group and an independent student t-test was performed (Advanced Statistics, SPSS 16). A two-tailed unpaired student's t-test was used to analyze differences between groups with * significant, if $p<0.05$; ** significant, if $p<0.01$; *** significant, if $p<0.001$.

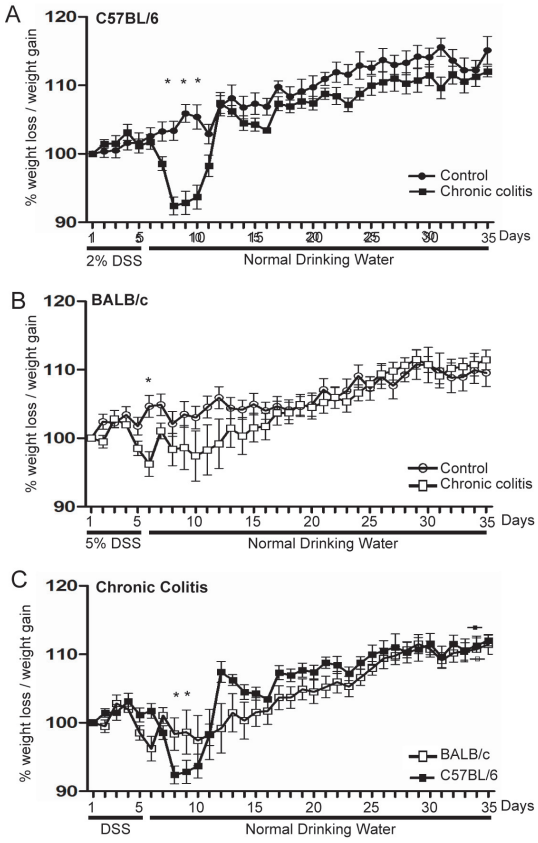


Figure 1. Daily changes in body weight of DSS- induced colitis in C57BL/6 and BALB/c mice.

Average of daily weight +/– SEM of control animals is indicated with circles and of animals with colitis with squares. C57BL/6 mice are indicated with black circles and squares and BALB/c mice are indicated with white circles and squares. Daily weight graphs are shown for C57BL/6 control animals and C57BL/6 chronic colitis animals (A), BALB/c control and BALB/c chronic colitis animals (B), and for C57BL/6 chronic colitis mice compared to BALB/c chronic colitis mice (C). C57BL/6 chronic colitis mice received 2% DSS in drinking water for the first five days of the experiment followed by a thirty day period on normal drinking water. BALB/c chronic colitis mice received 5% DSS in drinking water for the first five days of the experiment followed by a 30 day rest period on normal drinking water. Both control groups included in the experiment received normal drinking water without DSS throughout the experiment.

Results

BALB/c mice are more resistant to DSS-induced colitis than C57BL/6 mice.

To further investigate the differences observed in BALB/c mice versus C57BL/6 mice in developing DSS-induced colitis we first needed to establish DSS-induced colitis in both mouse strains in our own facilities. C57BL/6 mice were given 2% DSS in drinking water for five days followed by a 30 day consecutive period on normal drinking water. Daily weight graphs showed that C57BL/6 mice started to lose weight on day 6 with a maximum weight loss of 7.6% on day 8, which was followed by a recovery phase (Figure 1A). DSS colitis in BALB/c mice was induced in a similar manner. However, due to the fact that BALB/c mice were more resistant to developing colitis, we used 5% DSS in drinking water for five days, after having established that upon administration of 2%, 3%, 4% or 5% DSS, 5% DSS resulted in a disease score most comparable to C57BL/6 mice (data not shown). The kinetics of DSS-induced colitis was different in BALB/c mice compared to C57BL/6 mice. The daily weight graphs for BALB/c mice revealed that these mice started to lose weight on day 3 with a maximum weight loss of 3.8% which occurred on day 6 (Figure 1B). Thus, BALB/c mice tended to lose less weight at an earlier stage and recovered more quickly than C57BL/6 mice (Figure 1C). Macroscopic scoring of the colon revealed that, although BALB/c mice received a higher DSS % in the drinking water, C57BL/6 and BALB/c showed a comparable reduction in colon length during both acute colitis (day 7) and chronic colitis (day 35) (Figure 2A). Both inflammatory and diarrhea scores revealed a comparable form of disease in C57BL/6 and BALB/c mice (Figure 2B and C). Comparable results have been obtained by two other independent research groups using the same DSS administration protocol for these two mouse strains [3, 5].

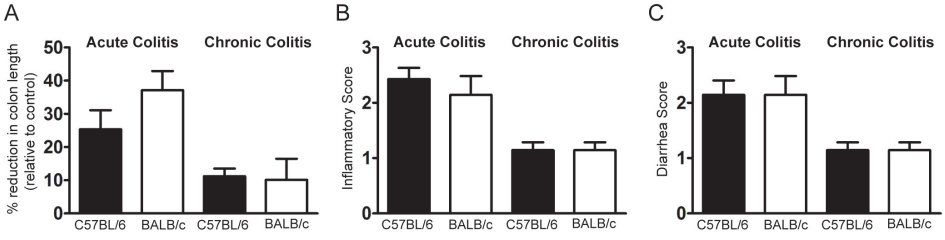
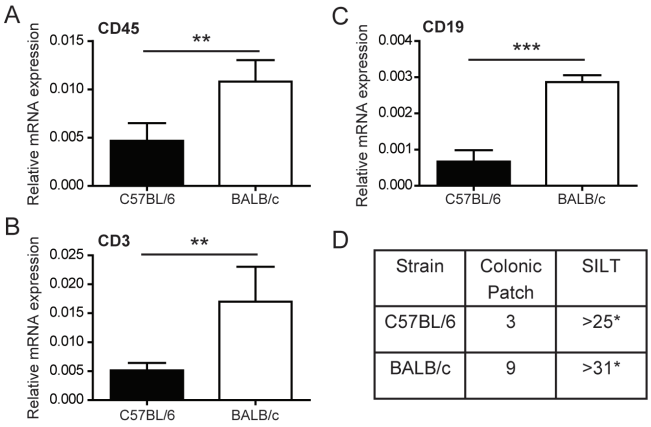


Figure 2. Macroscopic scores of DSS-induced colitis in C57BL/6 and BALB/c mice.

(A) Graphs shows percentage of reduction in colon length, which is used as an indication for the severity of the disease. (B) Graph shows the inflammatory score (0-4) which is also used as an indication of the severity of disease along with (C) the diarrhea scores (0-4). C57BL/6 mice are indicated by black bars and BALB/c mice are indicated by white bars. Mice in the acute phase are analyzed at day 7 of DSS-induced colitis and mice in the chronic phase are analyzed at day 35 of DSS-induced colitis. Bars indicated are represented by an average +/- SEM of seven animals per group.



Strain	Colonic Patch	SILT
C57BL/6	3	>25*
BALB/c	9	>31*

Figure 3. BALB/c mice contain more organized GALT structures in the healthy colon compared to C57BL/6 mice.

(A -C) Real time PCR analysis showing relative mRNA expression levels of (A) CD45, (B) CD19 and (C) CD3 detected in whole colon of healthy control C57BL/6 and BALB/c mice. Data represent the average per group +/- SEM. Per group 5 mice were used. (D) Table shows the number of SILTs and colonic patches (CPs) present in healthy colon of C57BL/6 and BALB/c mice (n=2 per group). In all figures, results obtained for C57BL/6 mice and BALB/c mice are indicated in black bars and white bars, respectively. Significant differences are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p < 0.001$).

BALB/c mice contain more colonic lymphoid tissue than C57BL/6 mice.

Since the observed differences in disease severity could be a result of a difference in the ability of the mucosal immune system to respond to tissue injury induced by DSS, we wanted to determine whether similar numbers of haematopietic cells were present within the colon. First, we determined the expression of CD45 (haematopietic marker) within healthy control colons of C57BL/6 and BALB/c mice. BALB/c control colons contained significantly more mRNA for CD45 than C57BL/6 control colons which indicated that more haematopietic cells were present within the non-inflamed BALB/c colon (Figure 3A). Further analysis of mRNA levels by real time PCR of the colons revealed an increased expression of both CD19 (B cells) and CD3 (T cells) in BALB/c control versus C57BL/6 control colons (Figure 3B and C). While these cells can be found as single isolated cells within the colon, most cells are present in organized lymphoid structures. Cryptopatches and isolated lymphoid follicles, referred to as immature and mature solitary isolated lymphoid tissue (SILTs) respectively, along with colonic patches (CPs) make up the organized GALT of the colon, which provide a microenvironment for immune responses to occur in an efficient manner [31, 32]. It has previously been shown that adult BALB/c mice contain more SILTs than C57BL/6 mice [33]. The increased mRNA expression levels for CD45, CD19 and CD3 could indicate that BALB/c mice have more organized GALT structures, i.e. colonic patches and SILTs, than C57BL/6 mice. To quantify this, we counted the total number of SILTs and CPs

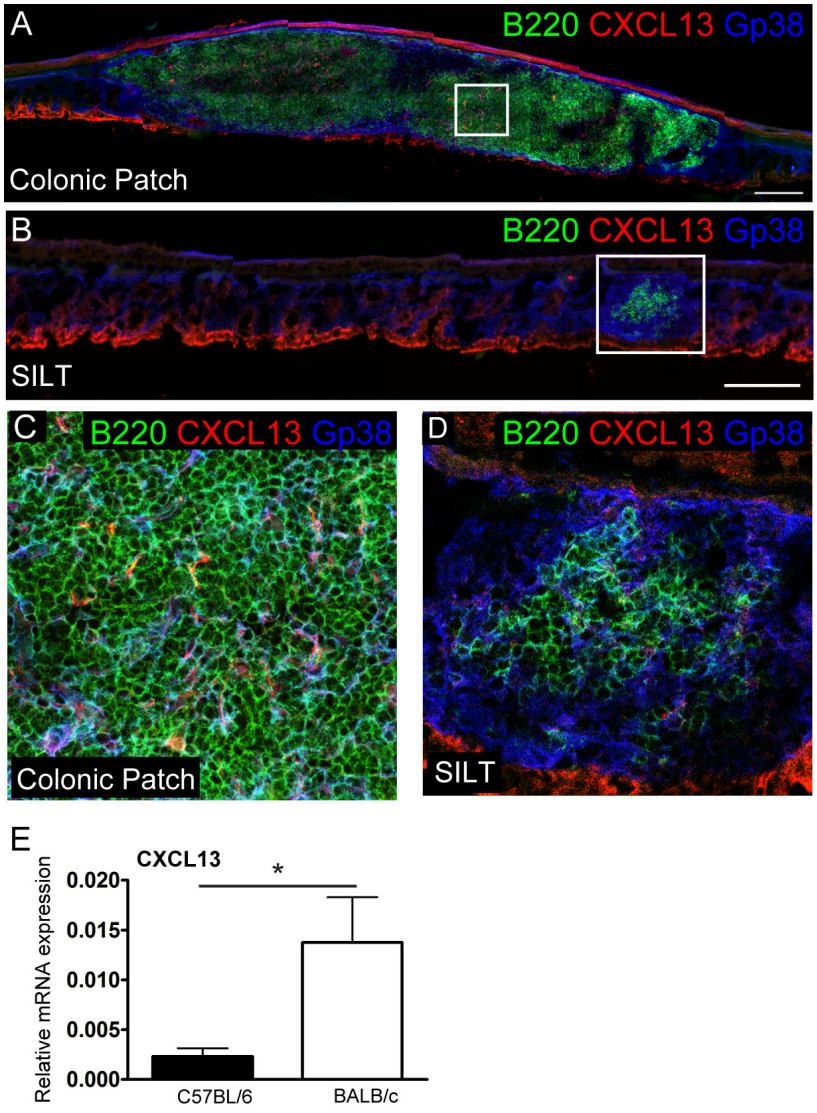


Figure 4. Colonic patches and SILTs contain CXCL13 producing stromal cells and consist mainly of B cells.

(A-D) Immunofluorescence analysis of control colons from BALB/c mice showing colonic patches (A and C) and SILTs (B and D). Representative overviews of colonic tissue taken with 20x magnification stitch picture function (A and B) and relevant area of interest (C and D). Pictures show organized GALT of the colon with stromal cells producing CXCL13 (B220 in green, CXCL13 in red and gp38 in blue, scale bars represent 250 µm). All pictures were taken on DM6000 Leica Immunofluorescence Microscope Leica (Leica Microsystems). (E) CXCL13 mRNA levels in colons of healthy BALB/c and C57BL/6 mice analyzed by real time PCR. C57BL/6 mice and BALB/c mice are indicated by black bars and white bars, respectively. Result represents the average per group +/- SEM. Per group 5 mice were used. Significant difference is indicated with * ($p<0.05$).

present in C57BL/6 versus BALB/c control colons by sectioning through the entire colon and screening every 20th section for the presence of organized GALT structures. Indeed, BALB/c control colons contained more CPs (9) and SILTs (>31) than C57BL/6 animals that have 3 CPs and >25 SILTs (Figure 3D).

Colonic patches and mature SILTs consist mainly of B cells, which cluster together to form follicles. The formation of these structures is principally controlled by stromal cells which express lymphotoxin β receptor (LTβR) [34-36]. The triggering of this receptor leads to the increased expression of a major B cell attracting chemokine, CXCL13, needed for SILT formation in the small intestine [37, 38]. The expression of CXCL13 might be restricted to mature SILTs and colonic patches and could serve as an additional measure for organized GALT within the colon. Indeed, upon analysis of CXCL13 expression within the tissue, we observed that CXCL13 colocalized with gp38+ stromal cells within B cell areas in mature SILTs and colonic patches (Figure 4A–D). To support our observation that more B cell follicles are present in BALB/c colons, we measured mRNA expression levels of CXCL13 in colons from healthy BALB/c and C57BL/6 mice with real time PCR. We detected higher expression levels of CXCL13 in BALB/c healthy colons when compared to C57BL/6, indicating that they express more CXCL13 needed for the formation of organized GALT present in the colons (Figure 4E).

Organized GALT structures contain IgA producing B cells and regulatory T cells.

The larger SILT structures in the small intestine have been shown to contain IgA+ B cells [33]. We have previously shown that SILTs and colonic patches in colons from healthy adult mice contain follicular dendritic cell networks with germinal centers, which should be capable to induce IgA isotype switching (Olivier *et al.* manuscript in preparation). We thus performed immunofluorescence stainings on colonic patches, which showed that colonic patches indeed consisted of distinct B cell follicles and T cell areas (Figure 5A). Within the B cell follicles, we could detect IgA+ B cells (Figure 5B).

Stainings of the T cell areas in colonic patches showed that these structures contained FoxP3 expressing regulatory T cells (Figure 5C). Thus, an increase of organized GALT structures may be beneficial for the host with regards to IgA production and Treg cell generation, which is of importance for maintaining tolerance to the microflora and suppressing unwanted immune responses.

During inflammation, BALB/c colons contain more haematopietic cells than C57BL/6 colons.

In the healthy setting, BALB/c colons contain more T and B cells than C57BL/6 colons, which may help to respond to DSS-induced colitis. We tested whether this difference was still present during inflammation at both the acute and chronic phase of the disease.

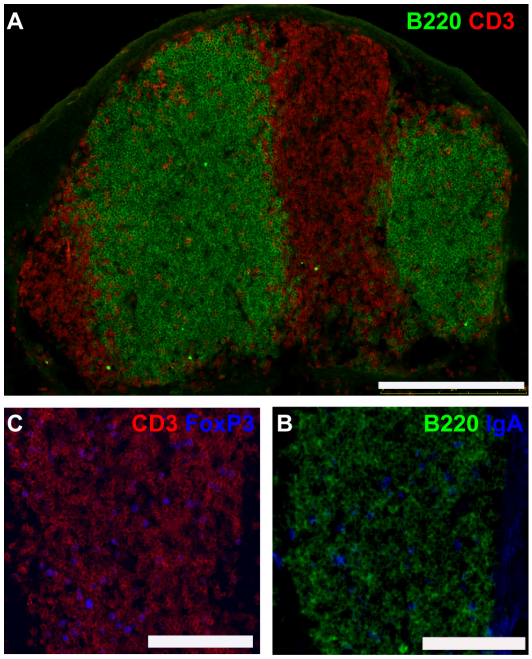


Figure 5. Organized GALT structures contain IgA producing B cells and regulatory T cells.
(A) Representative picture of immunofluorescence analysis of a colonic patch in a BALB/c mouse with both T cell and B cell areas. B220 is shown in green and anti-CD3 in red (scale bars = 250 µm). (B) Magnification of a B cell follicle which contains IgA producing B cells (B220 in green and IgA in blue, scale bars = 50 µm). (C) Magnification of the T cell area which contains regulatory T cells (CD3 in red and FoxP3 in blue. Scale bars = 50 µm). All pictures were taken on DM6000 Leica Immunofluoresence Microscope Leica (Leica Microsystems).

In the acute phase (day 7), real time PCR analysis revealed that also at this stage BALB/c mice have an increased mRNA expression of CD45, CD3 and CD19 when compared to C57BL/6 mice (Figure 6A–C). At this time point, BALB/c mice have already entered the recovery phase of colitis whereas C57BL/6 mice have not. Interestingly, during the chronic phase of inflammation, when both mouse strains are recovering from the disease, CD45 and CD3 mRNA analysis revealed no significant differences in expression levels in BALB/c and C57BL/6 mice. However, CD19 expression remained increased in BALB/c mice in the chronic phase of the disease. CXCL13, as a measure for B cells follicles present in the colon, also remained increased in both the acute and chronic phase in BALB/c mice (Figure 6D).

Another cell subset that is considered as protective for the intestinal integrity is formed by RORγt-expressing NKp46^{+} lymphoid tissue inducer (LTi) cells [25, 39, 40]. In addition, RORγt is also expressed by Th17 cells, which were also shown to have a protective effect [41-43]. To assess if RORγt-expressing cells, which could be beneficial in dealing with colitis, were increased in BALB/c mice, we measured expression of RORγt in colons with real time PCR. Indeed, the results show an increased mRNA expression of RORγt in BALB/c mice compared to C57BL/6 mice in the healthy, acute and chronic inflammatory setting indicating that BALB/c mice have an increase in a variety of immune cells which can be of benefit in dealing with colitis (Figure 6E).

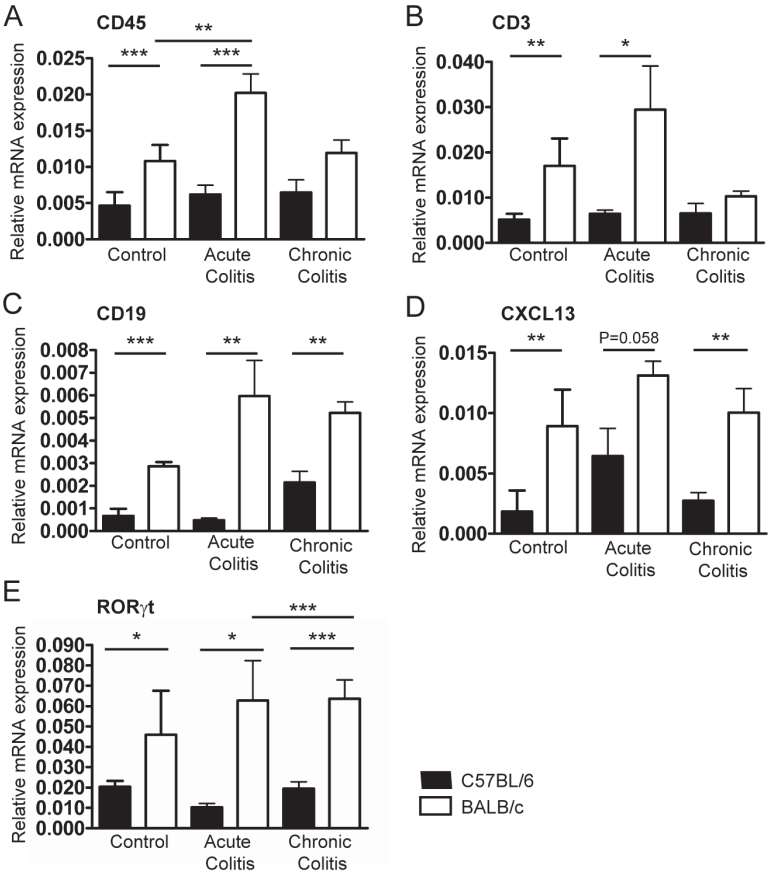


Figure 6. BALB/c colons contain more haematopoietic cells in both the acute and chronic inflammatory setting than C57BL/6 colons.

(A-D) Real time PCR analysis of colons in the control, acute (day 7) and chronic (day 35) inflammatory setting. Graphs show mRNA expression levels of CD45 (A; haematopoietic cells), CD3 (B; T cells) CD19 (C; B cells), CXCL13 (D) and RORγt (E). In all figures results obtained for C57BL/6 mice are indicated in black bars and BALB/c mice are indicated in white bars. All averages +/– SEM are representative of 5 mice per group. Significant differences are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p < 0.001$).

BALB/c control colons have higher levels of RALDH enzymes and more RA-mediated signalling.

The migration of lymphocytes to the intestine, along with the production of IgA and differentiation of regulatory T cells have all been shown to be under the influence of retinoic acid (RA), which is the active metabolite of vitamin A. The formation of RA is dependent on enzymes called retinaldehyde dehydrogenases (RALDH). Upon RA formation, RA binds nuclear retinoic acid receptors (RARs) which become active transcription factors that bind RAREs within the promoter region of their target genes. To see whether differences in RALDH expression between

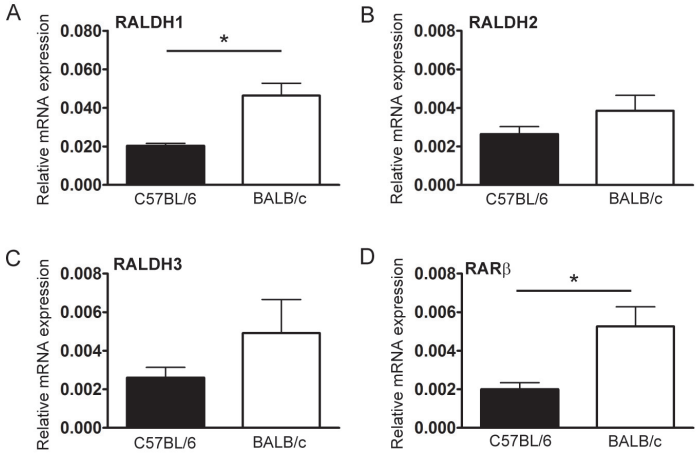


Figure 7. BALB/c mice have more retinoic acid-mediated signalling than C57BL/6 mice.
Real time PCR analysis of C57BL/6 and BALB/c control colons showing mRNA expression levels of RALDH1 (A), RALDH2 (B), RALDH3 (C) and RARβ (D). In all figures, results obtained for C57BL/6 mice are indicated in black bars and BALB/c mice are indicated in white bars. All averages +/– SEM are representative of 5 mice per group. Significant differences are indicated by * ($p<0.05$).

the two mouse strains existed, we analyzed the expression of the 3 isoforms of the RA converting enzymes, i.e. RALDH-1, -2 and -3 by real time PCR in healthy colons of BALB/c and C57BL/6 mice. Analysis revealed a significant increase in the expression of RALDH1 in BALB/c colons compared to C57BL/6 colons (Figure 7A). For the remaining two isoforms, i.e. RALDH-2 and -3, there was no significant difference observed (Figure 7B and C). To address whether indeed enhanced RA-mediated signalling occurred within BALB/c colons, we addressed expression levels of RARβ, which is a direct target gene of RA and shown to be an indicator of the level of RA-mediated signalling both *in vivo* and *in vitro* [44]. Analysis of RARβ mRNA levels showed that expression levels for RARβ were significantly increased in control colons of BALB/c mice when compared to C57BL/6 mice, indicating that indeed more RA-mediated signalling takes place within the colon of BALB/c mice when compared to C57BL/6 (Figure 7D).

Vitamin A deficiency increases the severity of chronic colitis.

To assess if the level of RA synthesis indeed correlates with the severity of colitis, mice were raised either on a diet containing a normal vitamin A concentration or on a diet deficient in vitamin A. Subsequently, colitis was induced and their health status was monitored. Vitamin A deficient mice suffered from a more severe colitis than mice on a vitamin A control diet as indicated by the weight loss and diarrhea (Figure 8A and B). In fact, 5 of the 7 animals in these experiments had to be euthanized before the end of the experiment because of severe disease.

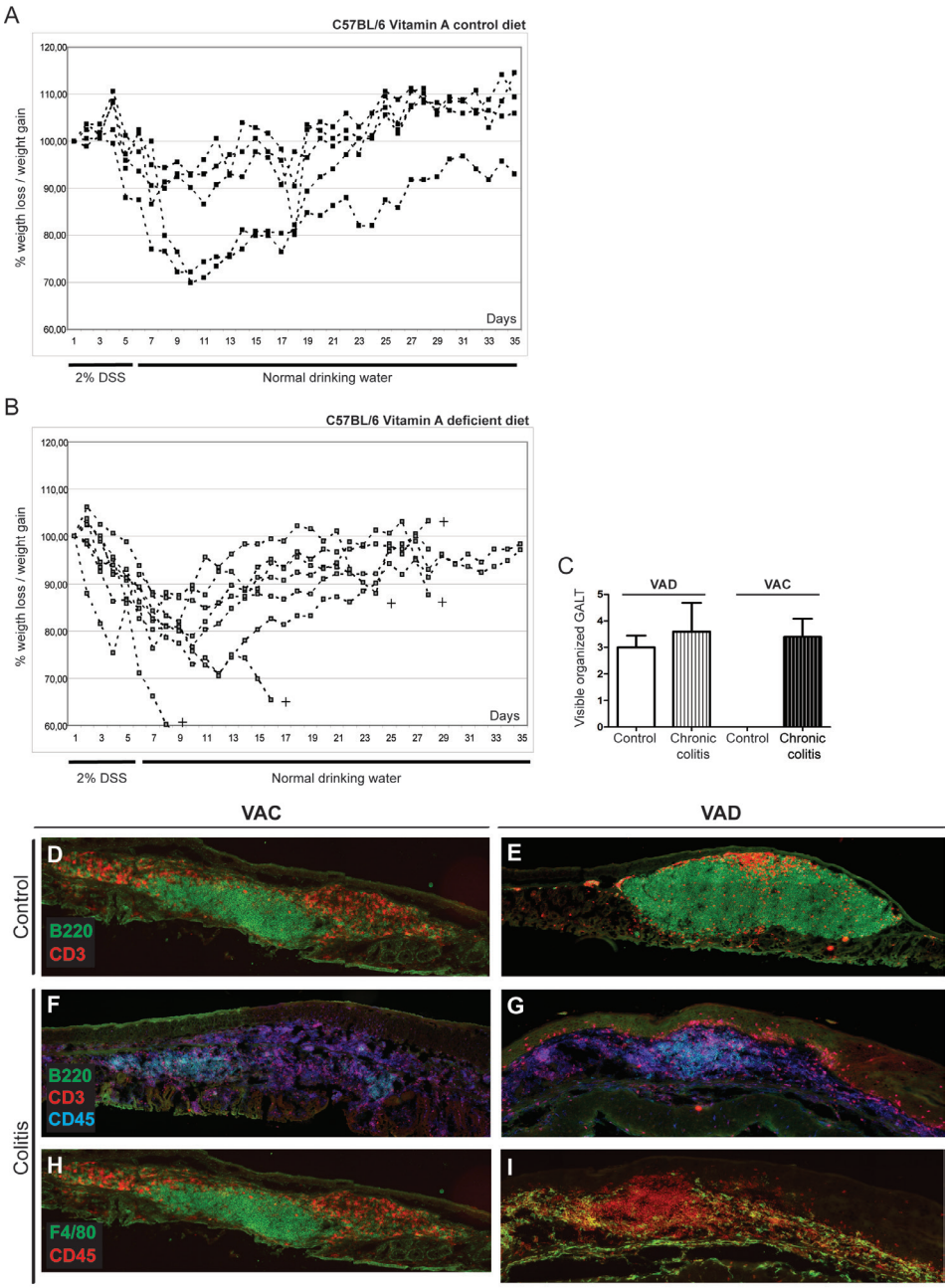


Figure 8. Vitamin A deficiency increases the severity of DSS-induced colitis.

Daily weight graphs of animals on a vitamin A control diet (A) and a vitamin A deficient diet (B) given 2% DSS in drinking water for the first five days of the experiment followed by a 30 day period on normal drinking water. Number of organized GALT structures was counted by macroscopic scoring (C). Colons of vitamin A control (D, F) and vitamin A deficient (E, G) without or with DSS administration were analyzed by H&E stainings at 35 days after initiation of DSS administration.

The remaining two animals only returned to their starting weight at the end of the experiment. Macroscopic inspection of the colons showed large lymphoid structures in vitamin A deficient control mice, which were not visible in control mice on vitamin A sufficient diet (Figure 8C). Upon histological examination these larger lymphoid structures in colons of vitamin A control mice were confirmed (Figure 8D and 8E). DSS administration resulted in histological signs of colitis in both vitamin A deficient and vitamin A control animals with more profound cellular infiltrates and bowel thickening in vitamin A deficient mice when compared to mice on vitamin A control diets (Figure 8F and G). These results suggested that vitamin A deficiency already leads to an enhanced cellular influx into the colon of untreated vitamin A deficient mice, while upon DSS treatment the animals get a more severe colitis than animals on a vitamin A control diet. This confirms our hypothesis that dietary intake of vitamin A is indeed needed for a protective effect on inflammatory disease within the colon.

Discussion

BALB/c mice suffer from less severe DSS-induced colitis compared to C57BL/6 mice and tend to recover more quickly after the withdrawal of DSS. Here we show that in the healthy setting BALB/c mice have more organized GALT structures in the colon and this may be responsible for the less severe colitis and the quicker recovery observed in this mouse strain. It has been shown that inducible bronchus associated lymphoid tissue (iBALT) in the lung has a protective effect and helps animals clear influenza virus at an enhanced rate [45, 46]. It has also been shown that these iBALT structures contain germinal centers, which act as additional ectopic sites that promote immunoglobulin class switching and thus promote IgA production, which aids the host in clearing infection [45]. In the context of the intestine, Lorenz and Newberry have shown that larger SILTs containing B cells play a role in the production of antigen-specific IgA [47]. Peyers' Patches of the small intestine have also been shown to be involved in antigen-specific IgA production [48] and in this paper we show that colonic patches, which are the counterpart of Peyers' Patches, present in the colon also contain B cells which are capable of IgA production. Intriguingly, it has also been shown that RA is needed for immunoglobulin class switching by B cells to be able to produce IgA [21, 49]. RA has also been shown to aid in the differentiation of regulatory T cells, which are known to suppress inflammation, and the induction of gut homing molecules needed for these cells to return to the intestines [18-20]. In this paper we have shown that $FoxP3^+$ regulatory T cells are largely present in colonic patches. Organized GALT structures, i.e. SILTs and colonic patches containing IgA-producing B cells and regulatory T cells, provide a micro environment in which numerous beneficial immune responses may occur, which may be responsible for the decreased severity of colitis in BALB/c mice.

RA is not only involved in the production of IgA by B cells and the differentiation and gut homing of regulatory T cells to the intestine but can also potentially play a role in the formation of organized GALT. It has been shown that RA can induce CXCL13 expression in stromal organizer cells, which is the initial event in the formation of peripheral lymph nodes during embryonic development [24]. In the context of the adult intestine, transgenic overexpression of CXCL13 in epithelial cells leads to an increase of both B cells and LTi cells and promotes the formation of SILTs [25]. Considering that adult BALB/c mice have more organized GALT in the healthy setting, in combination with higher RA-mediated signalling and higher levels of CXCL13, it is tempting to speculate that the higher levels of RA-mediated signalling in BALB/c mice is responsible for the observed increase in organized GALT in these animals. Interestingly, we show that in addition to more organized GALT structures containing T and B cells, BALB/c mice express higher levels of RORγt. RORγt is expressed by various cell subsets in the intestine i.e. LTi cells, $NKp46^+$ cells and Th17 cells [41-43, 50]. All of these cell types have been shown to have beneficial or protective effects in helping the host cope with infection. It is likely that also the presence of these cells will further aid in the resistance that BALB/c mice show towards DSS-induced colitis. Whether RA-mediated signalling is involved in their existence within the intestine will need further study. It has been shown that RA can mediate the balance between pro- and anti-inflammatory responses [51]. In the context of TNBS colitis, it has been shown that the administration of RA ameliorates colitis by shifting the balance from a Th1 to a Th2 response [52]. Furthermore, there is evidence to support the fact that RA can shift the Treg/Th17 balance in both TNBS colitis and human ulcerative colitis [53]. Confirming the effects of vitamin A and RA in our study, we show that the removal of vitamin A from the diet, and thus a decrease in RA levels, gives rise to a more severe form of colitis. The fact that BALB/c mice express higher levels of RALDH enzymes and display higher RA-mediated signalling could serve as an explanation as to why BALB/c mice are more resistant to DSS-induced colitis than C57BL/6 mice.

Taken together, BALB/c mice have more organized GALT structures, containing more T cells, B cells and regulatory T cells, and display higher RA-mediated signalling when compared to C57BL/6 mice. It is tempting to speculate that inherent differences in RA-mediated signalling are responsible for this increase, and could explain why BALB/c mice suffer from less severe colitis and have a quicker recovery than C57BL/6 mice. Intriguingly, in the context of the human form of disease, it has been shown that IBD patients frequently suffer from vitamin A deficiency [54]. This raises the attractive hypothesis that administration of vitamin A and or RA to patients suffering from IBD could serve as an effective treatment or alleviation of disease in these patients.

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7

Retinoic acid promotes lymphoid tissue inducer cell differentiation during embryonic lymph node development

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Abstract

The vitamin A metabolite retinoic acid (RA) has been reported to have numerous effects on haematopoietic cells. Here we demonstrate that RA promoted differentiation of haematopoietic LTi cells during embryonic development. Both addition of RA to embryonic cells *in vitro* as well as supplementing pregnant mice with RA *in vivo,* mediated the differentiation of haematopoietic precursors from $CD4^-$ IL-$7R\alpha^+$ to $CD4^+$ IL-$7R\alpha^+$ mature LTi cells. On the contrary, withdrawal of vitamin A from pregnant mice resulted in a significant decrease of mature LTi cells. Furthermore, mice with enhanced vitamin A metabolism displayed more mature LTi cells and larger mucosal lymphoid organs. Therefore, our data showed that RA mediates local differentiation of LTi cells and that the availability of RA has a measurable effect on haematopoietic differentiation during embryonic development.

Introduction

Development of secondary lymphoid organs like Peyers' patches (PPs) and mesenteric lymph nodes (MLNs) involves clustering of haematopoietic lymphoid tissue-inducer (LTi) cells, characterized as $CD45^+$ $CD4^+$ $CD3^-$ IL-7Rα^+ RORγt^+, with $VCAM^+$ mesenchymal stromal organizer cells [1-3]. At specific locations in the embryo, LTi cells expressing membrane bound lymphotoxin-$\alpha\beta$, cluster together and interact with the lymphotoxin-β receptor (LTβR) on stromal organizer cells [4]. LTβR triggering causes stromal organizer cells to synthesize chemokines and adhesion molecules that attract and retain more LTi cells [5]. This initiates a chain of events that involves further accumulation and signalling of LTi cells and organization of the cell clusters into complete lymph nodes.

A number of factors are crucial for lymph node development by promoting the differentiation and survival of LTi cells. Mice deficient in the retinoic orphan receptor RORγt or the inhibitor of transcription Id2 lack LTi cells and consequently, the development of lymph nodes is disturbed [6-8]. In addition, deletion or inhibition of the interleukin-7 receptor α (IL-7Rα) also affected lymph node development [6, 9], while enhanced expression of IL-7 promoted the survival and accumulation of LTi cells and resulted in the formation of larger lymph nodes as well as ectopic lymph nodes [10]. Furthermore, TRANCE deficient mice showed defective lymph node formation and reduced numbers of LTi cells which both could be rescued by transgenic overexpression of TRANCE [11]. Therefore, the formation of both normal and ectopic lymphoid organs can be regulated by controlling LTi cell numbers.

IL-7Rα and CD4 are expressed at the final differentiation stages of the LTi lineage [4], but not all LTi cells present within the lymph node anlagen are $CD4^+$ [5, 6, 12, 13]. This suggests that $CD45^+$ IL-7Rα^+ $CD4^-$ $CD3^-$ LTi cells form the precursors of mature $CD45^+$ IL-7Rα^+ $CD4^+$ $CD3^-$ LTi cells and probably differentiate locally into mature LTi cells [5, 13, 14].

Vitamin A plays a critical role in the development and function of the immune system. Previously, we have shown that the initiation of embryonic lymph node development is controlled by the vitamin A metabolite retinoic acid (RA). RA induced expression of CXCL13, which is necessary for the first clustering of LTi cells [12]. Nerve fibers adjacent to the location of lymph node development expressed RALDH enzymes necessary for conversion of vitamin A into RA [12] and are therefore most likely the source for RA and responsible for CXCL13 induction in stromal organizer cells. Furthermore, in the adult, differentiation of mucosal DCs in the intestinal lamina propria has been shown to involve RA (Chapter 4 and [15, 16]). Moreover, RA coordinates the function of lymphocytes within the mucosal immune system at multiple levels, such as regulating gut-homing molecule expression on T and B cells [17, 18] and the balance between regulatory T cells and Th17 cells [19-21].

These studies demonstrate that RA is of vital importance during embryonic development of the immune system as well as proper functioning of the immune system in the adult by affecting the differentiation and function of multiple haematopoietic cell subsets. Since LTi-like cells are also identified in adult intestines and thus associated with a retinoic acid-rich environment [22, 23], we hypothesized that RA may influence haematopoietic LTi differentiation in the embryo. Here we demonstrate that RA promoted the differentiation of LTi cells from $CD4^-$ IL-$7R\alpha^+$ to $CD4^+$ IL-$7R\alpha^+$ LTi cells both *in vivo* and *in vitro*. Supplementation of pregnant mice with RA skewed the differentiation of haematopoietic precursors towards the mature LTi phenotype in the embryo. On the contrary, embryos from mothers that received vitamin A deficient diet showed a reduced LTi cell differentiation towards the mature LTi phenotype. We could correlate differences in vitamin A metabolism between mouse strains with the presence of more mature LTi cells and larger mucosal lymphoid organs. Therefore, our data supports the hypothesis that RA regulates local differentiation of LTi cells, suggesting that the level of RA production during pregnancy can affect the formation of lymphoid tissue in the offspring.

Materials and methods

Animals

C57BL/6 mice and BALB/c mice aged 10-14 weeks were obtained from Charles River (Charles River, Maastricht, The Netherlands) and kept at our own facilities under standard animal housing conditions. The Animal Experiments Committee of the VU Medical Center approved all of the experiments described in this study. To obtain vitamin A deficient (VAD) and vitamin A control (VAC) embryos, pregnant C57BL/6 mice either received a chemically defined diet that lacked vitamin A (the modified AIN-93M feed, MP Biomedical, Solon, Ohio, USA), or that contained vitamin A (2800 IU/kg in the modified AIN-93M feed, MP Biomedical) from E8.5 until sacrifice at E18.5.

To generate RA supplemented embryos, pregnant C57BL/6 mice were provided with 250µg retinoic acid/g food (RA; Sigma-Aldrich, Zwijndrecht, The Netherlands) from E10.5 until sacrifice at E13.5 [24, 25]. RA was dissolved in 100% ethanol at 5 mg/ml. Either 250 µg/g chow was made by diluting 8 ml stock to 100 ml with water to which 60 g of ground chow was added. Food was refreshed every day until sacrifice.

In vitro retinoic acid stimulation

For *in vitro* stimulation, embryonic cells were harvested as described before [12]. In short, E13.5 C57BL/6 embryos were used and all extremities including the

head above the eyes and internal organs including haematopoietic cell containing organs and mesenteric lymph nodes (MLNs) were removed. The tissue was digested with 0.5 mg/ml Blendzyme 2 (Roche, Penzberg, Germany), 0.2 mg/ml DNase-I (Roche) in PBS for 15 min at 37˚C while constantly stirring. Cell suspensions were washed with RPMI (Invitrogen, Breda, The Netherlands), supplemented with 2% heat-inactivated FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were allowed to adhere for 2 hours at 37˚C. For RA stimulation experiments, all-trans retinoic acid (RA, Sigma-Aldrich, Zwijndrecht, The Netherlands, dissolved as 10 mM in 100% ethanol) was added at 100 nM. After incubation for 24 hours at 37˚C and 5% CO_2, cells were analyzed by flow cytometry.

Flow cytometry

MLNs were isolated from VAD and VAC embryos at E18.5, or from C57BL/6 and BALB/c embryos at E18.5. Embryos from RA supplemented mothers were collected at E13.5 and dissected as described above. Single cell suspensions were made by cutting tissues with scissors, followed by digestion at 37ºC for 20 min, using 0.5 mg/ml Blendzyme 2 (Roche) and 0.2 mg/ml DNase-I (Roche) in PBS while constantly stirring. Cell clumps were removed by pipetting the cells over a nylon mesh. Lymph node cells were washed and resuspended in PBS with 2% NBCS. Subsequently, cells were stained with anti-IL-7R antibody (anti-CD127, clone A7R34, eBioscience, Immunosource, Halle-Zoersel, Belgium), Alexa fluor 488 (Invitrogen, Breda, the Netherlands) conjugated anti-CD45 (clone MP33), Alexa fluor 488 conjugated anti-CD4 (clone GK1.5), Alexa fluor 488 conjugated anti-CD3 (clone 145-2C11, eBioscience), PE conjugated anti-CD4 (clone GK1.5, eBioscience), PE-Cy7 conjugated anti-CD45 (clone 30-F11, eBioscience) and with Sytox Blue (Invitrogen) to discriminate between live versus dead cells. Secondary antibody used was Alexa fluor 647 conjugated goat anti-rat antibody (Invitrogen). Cells were analyzed with a Cyan ADP flow cytometer (Beckman Coulter, Woerden, The Netherlands).

Macroscopic analyis of lymph nodes and Peyer's Patches

MLNs were carefully dissected from 10-14 week old BALB/c and C57BL/6 mice and the length of the mesenteric lymph node string was measured. Peyer's patches (PPs) were excised and the number of PPs per small intestine were counted. The weight of MLNs and PPs was determined.

Statistics

The data shown in figure 1B and 1C were analyzed with a paired Student's *t*-test. A two-tailed unpaired Student's *t*-test was used for all other analyses.

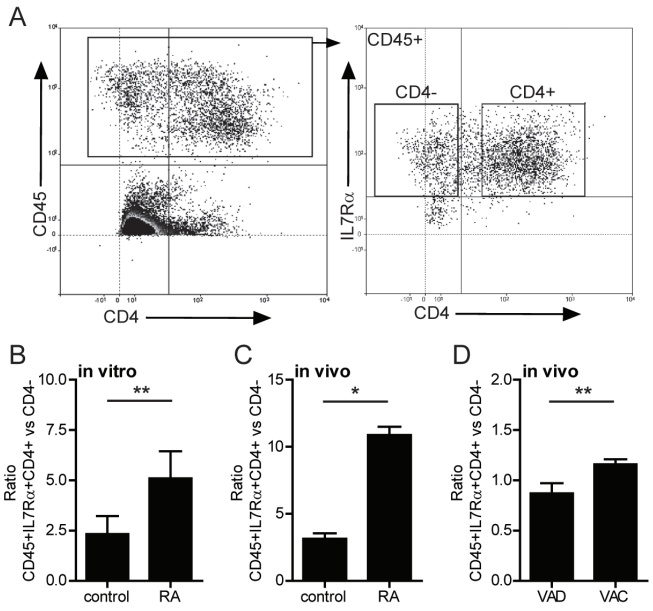

Figure 1. RA promotes differentiation of precursor LTi cells to mature LTi cells.

(A) Shown are representative flow cytometry plots of CD45, CD4 and IL-7Rα expression by PLN enriched embryonic cells from E13.5 C57BL/6 embryos. Boxes indicates $CD45^+$ gate and $CD4^+$ and $CD4^-$ gate for calculations of LTi ratios shown in B, C and D. (B) *In vitro* cultures of PLN enriched embryonic cells from E13.5 C57BL/6 embryos stimulated with 100 nM RA for 24 hours. 3 individual experiments with n=7. (C) PLN enriched embryonic cells (E13.5 C57BL/6) from pregnant mice supplemented with 250 µg RA/g food. 2 individual experiments with n=9. (D) MLN suspensions from vitamin A deficient (VAD) and vitamin A control (VAC) embryos at E18.5. 2 individual experiments with n=10. Cells were analyzed for expression of CD45, CD3, IL-7Rα, and CD4. The ratio of the percentage of $CD45^+$ $CD3^-$ IL-7Rα^+ $CD4^+$ cells over $CD45^+$ $CD3^-$ IL-7Rα^+ $CD4^-$ cells was calculated. Data represent average ratio ± SEM. Significant differences are indicated as * (p=3.25E-08) or ** (p=0.02).

7

Results

Retinoic acid influences differentiation of haematopoietic LTi cells.

Since RA is involved in haematopoietic differentiation [15, 18, 20, 26, 27] and important for the induction of CXCL13 in mesenchymal cells of lymph node anlagen [12], we hypothesized that RA could also influence haematopoietic LTi differentiation in developing lymph nodes. To test this, we made cell suspensions from embryonic tissues enriched for peripheral lymph node anlagen [12]. After adherence of mesenchymal cells, RA was added to these cultures and 24 hours later the percentages of precursor LTi and mature LTi cells were analyzed by flow cytometry. Representative flow cytometry plots show gating for $CD45^+$ cells and expression of CD4 and IL-7Rα (Figure 1A). Since $CD45^+$ $CD3^-$ IL-7Rα^+ $CD4^-$ LTi

cells represent immature LTi cells and form the precursors to CD45$^+$ CD3$^-$ IL-7Rα^+ CD4$^+$ mature LTi cells [5, 13, 14], we calculated the ratio of CD45$^+$ IL-7Rα^+ CD4$^+$ mature LTi cells over CD45$^+$ IL-7Rα^+ CD4$^-$ precursor LTi cells. Upon RA stimulation, the ratio of mature LTi cells versus precursor LTi cells was significantly skewed towards the mature CD4$^+$ population (Figure 1B). To confirm *in vivo* that indeed RA can mediate the differentiation of LTi cells, we provided pregnant mice with RA in their food, starting at E10.5 [24, 25], 3 days before sacrifice at E13.5. After isolating peripheral lymph node anlagen enriched embryonic tissue, cells were analyzed by flow cytometry for LTi cells and their precursors. We observed that *in vivo* supplementation of RA dramatically increased the mature CD4$^+$ population (Figure 1C). Therefore, we conclude that RA can mediate the differentiation of immature CD45$^+$ CD3$^-$ IL-7Rα^+ CD4$^-$ LTi cells towards mature CD45$^+$ CD3$^-$ IL-7Rα^+ CD4$^+$ LTi cells.

Absence of vitamin A in diet of pregnant mice affects LTi cell differentiation in the offspring.

Since we observed that RA supplementation to pregnant females increased the differentiation of LTi cells during embryonic development, we reasoned that reducing the availability of RA should result in decreased embryonic LTi cell differentiation. To test this hypothesis, we provided vitamin A, from which RA is metabolized, deficient food (VAD) to pregnant mice at E8.5 and isolated the mesenteric lymph nodes (MLNs) at E18.5. VAD diet gradually depletes liver stores of pregnant mothers and thus reduces RA availability during embryonic development. FACS analysis on digested E18.5 MLNs indeed showed that deprival of vitamin A led to a significant decrease of CD45$^+$ IL-7Rα^+ CD4$^+$ mature LTi cells (Figure 1D).

Differentiation towards mature LTi cells is enhanced in BALB/c mice.

Since we have observed that BALB/c and C57BL/6 mice differ in vitamin A metabolism and RA-mediated signalling (Chapter 5)and that RA increases the amount of mature LTi cells, we reasoned that the amount of mature LTi cells might be different in BALB/c versus C57BL/6 mice. Therefore, the developmental stage of LTi cells in E18.5 MLNs derived from either BALB/c or C57BL/6 embryos was determined by flow cytometry. The percentage of CD45$^+$ haematopoietic cells in these MLNs was similar in both mouse strains (Figure 2A). At this age, few B cells and hardly any T cells were present within MLNs (4.4% ± 1.4 and 3.0% ± 0.6 of CD45$^+$ cells in C57BL/6 and BALB/c, respectively) and therefore nearly all of the CD45$^+$ cells were LTi cells. Next, we analyzed the developmental stage of LTi cells by measuring CD4 expression on CD45$^+$ CD3$^-$ IL-7Rα^+ LTi cells. C57BL/6 mice contained more immature CD4$^-$ IL-7Rα^+ LTi cells (Figure 2B, left) and less mature CD4$^+$ IL-7Rα^+ LTi cells (Figure 2B, right) in MLNs when compared to BALB/c

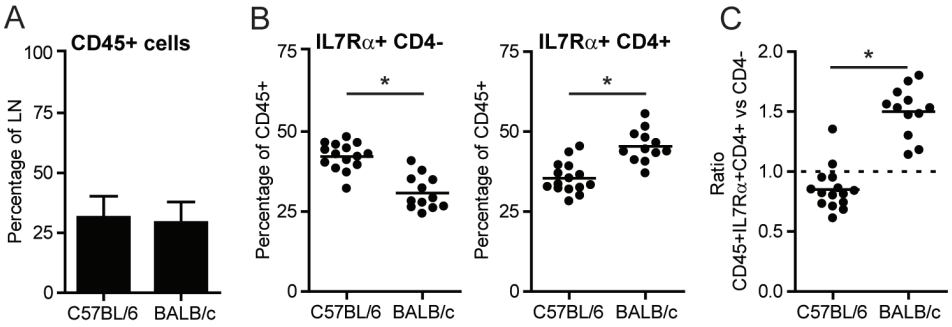

Figure 2. Differentiation of LTi cells is increased in BALB/c mice.

MLNs were isolated from C57BL/6 and BALB/c mice at day of birth. MLN cell suspensions were analyzed for expression of CD45, CD3, IL7Ra, and CD4. (A) Data represent the average percentage ± SD of $CD45^+$ cells in C57BL/6 and BALB/c MLNs. (B) Shown is the average percentage of $CD45^+$ cells that are $IL7R\alpha^+$ $CD4^-$ (left), and are $IL7Ra^+$ $CD4^+$ (right) of individual MLNs. (C) The ratio of the percentage of $IL7Ra^+$ $CD4^+$ cells over $IL7Ra^+$ $CD4^-$ cells of individual animals was calculated (right). A total of 15 C57BL/6 derived from 3 different litters and 12 BALB/c mice derived from 2 different litters were used for analysis in two separate experiments. Significant differences are indicated by * ($p<0.0001$).

mice. As a result, the ratio of mature $CD4^+$ IL-$7R\alpha^+$ LTi cells over precursor $CD4^-$ IL-$7R\alpha^+$ LTi cells was significantly lower in C57BL/6 MLNs than in BALB/c MLNs. And thus, enhanced vitamin A metabolism and RA-mediated signalling in BALB/c mice correlated with enhanced LTi differentiation towards more mature LTi cells (Figure 2C).

Transgenic overexpression of IL-7 has been shown to lead to increased numbers of LTi cells resulting in enlarged Peyers Patches (PPs) and MLNs and ectopic lymph nodes [10]. We described in chapter 6 that the colon of BALB/c mice contained much more isolated lymphoid follicles and cryptopatches than C57BL/6 mice. In addition, we observed that PPs and MLNs in BALB/c mice were larger than those in C57BL/6 mice. To quantify this we investigated the size of mucosal secondary lymphoid organs in both mouse strains by measuring length and weight of both MLNs and PPs. Representative pictures show PPs on small intestines of C57BL/6 and BALB/c mice (Figure 3A) and dissected MLNs from both mouse strains (Figure 3B). Upon measurement, MLNs from BALB/c mice were significantly larger than from C57BL/6 mice and by weight BALB/c MLNs were significantly heavier when compared to C57BL/6 MLNs (Figure 3C). We could detect slightly more PPs in BALB/c small intestines than in C57BL/6 small intestine (Figure 3D, left). Moreover, the weight of pooled PPs per BALB/c mouse was increased more than 2-fold compared to C57BL/6 PPs (Figure 3D, right). These data indicate that the generation of higher numbers of mature LTi cells in BALB/c mice may consequently not only lead to the formation of larger lymphoid tissues but also to more lymphoid tissues within the mucosal immune system.

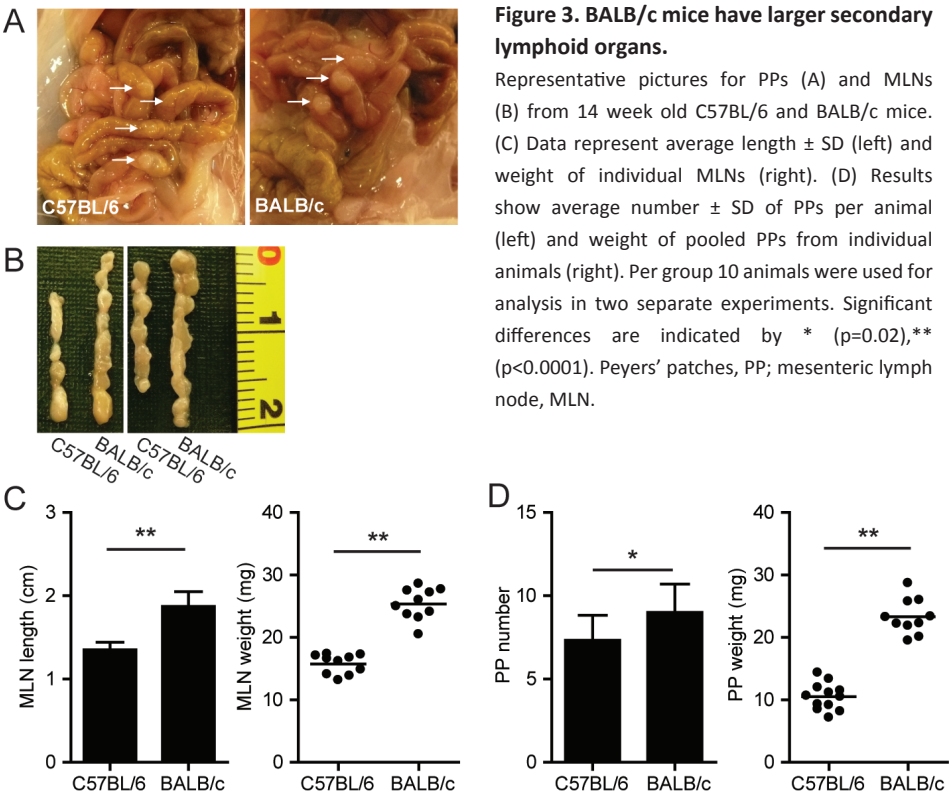

Figure 3. BALB/c mice have larger secondary lymphoid organs.

Representative pictures for PPs (A) and MLNs (B) from 14 week old C57BL/6 and BALB/c mice. (C) Data represent average length ± SD (left) and weight of individual MLNs (right). (D) Results show average number ± SD of PPs per animal (left) and weight of pooled PPs from individual animals (right). Per group 10 animals were used for analysis in two separate experiments. Significant differences are indicated by * ($p=0.02$),** ($p<0.0001$). Peyers' patches, PP; mesenteric lymph node, MLN.

Discussion

The vitamin A metabolite RA has numerous effects on haematopoietic cells, such as the migration of lymphocytes towards the intestines [17, 18] and the development of regulatory T cells [19-21]. Here we have shown that increased RA availability results in enhanced LTi differentiation and the development of more mucosal lymphoid tissue. By using several approaches, we showed that RA stimulation resulted in more mature LTi cells, defined as IL-7Rα^+ CD4$^+$, at the expense of the IL-7Rα^+ CD4$^-$ precursor population. This differentiation was affected by withdrawal of vitamin A from pregnant females, resulting in a significant decrease of mature LTi cells. Moreover, by studying mouse strains that differ in vitamin A metabolism we discovered that differentiation of LTi cells is enhanced in mice with higher RALDH expression and RA-mediated signalling (Chapter 5). This increased RA-mediated signalling is most likely responsible for increased mature LTi numbers in the BALB/c mice. In conclusion, RA is important for the differentiation of the progenitor LTi cells towards their final phenotype and is therefore essential for embryonic lymph node development.

Previously, we have shown that nerve fibers adjacent to lymph node anlagen express RA-metabolizing RALDH2 enzymes and are likely producers of RA leading to the induction of CXCL13 expression in mesenchymal cells [12]. RA-induced CXCL13 attracts CXCR5 expressing LTi progenitor cells towards the anlagen. Additionally, LTi differentiation towards the mature LTi phenotype might also be promoted directly by RA derived from adjacent nerve fibers. In that case, nerve-derived RA will not only affect LTi cell attraction to lymph node anlagen, but will also promote LTi cell differentiation and survival. Alternatively, in addition to CXCL13 induction, nerve-derived RA might induce RALDH expression in mesenchymal stromal organizer cells within the lymph node anlagen. We have shown that RALDH enzyme expression was induced in RA stimulated adult stromal cells *in vitro* (Chapter 4). Within lymph node anlagen, stromal organizer cells and LTi cells are in close contact and stromal organizer cells might provide LTi cells with RA for their differentiation upon RALDH induction.

Multiple factors are required for LTi cell differentiation like RORγt, TRANCE, and Id2 [6, 8, 11]. It remains unknown how RA is involved in LTi cell differentiation. Possibly RA affects LTi differentiation by regulating the expression levels of one or more of these factors. A good candidate is the helix-loop-helix repressor protein Id2. Id2 is an early regulator of LTi cell differentiation [8]. It inhibits T and B cell development [28] and promotes the differentiation of haematopoietic precursors into NK cells and LTi cells [29]. It has been shown that Id2 is a RA-responsive gene [30], thus RA might control LTi cell differentiation by positively regulating expression levels of Id2 proteins during lymph node development.

BALB/c mice showed enhanced LTi differentiation and displayed larger mucosal lymphoid tissues when compared to C57BL/6 mice. Others have reported that the number of LTi cells correlated with the amount of lymphoid tissue [10]. This allows for the hypothesis that maternal vitamin A metabolism may promote mucosal lymphoid tissue development in the fetus. Therefore, the amount of RA produced and the level of RA-mediated signalling may be instrumental for the development of the mucosal immune system during embryonic development. Since the production of RA is mostly dependent on conversion by RALDH enzymes, the levels of RALDH expression will determine the efficiency of LTi cell differentiation. It is therefore of importance to know why BALB/c and C57BL/6 mice differ in RALDH enzymes levels, and thus to dissect what controls RALDH expression levels within the developing embryo and pregnant mothers. This could potentially lead to therapeutic applications to stimulate the development of the mucosal immune system in the unborn child.

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8

Summary and discussion

Index

Summary

Mucosal immune system

The mucosal immune system protects us from harmful pathogens and at the same time maintains tolerance to harmless food antigens and commensal bacteria. The vitamin A metabolite retinoic acid (RA) is crucial for the mucosal immune system in maintaining this balance. In studies described in this thesis, several beneficial effects of RA have been investigated. We demonstrated that intestinal epithelial cells are involved in educating dendritic cells (DCs) to acquire a mucosal phenotype by upregulating RALDH enzyme expression. By omission of vitamin A from the diet we showed that dietary vitamin A is mandatory for RALDH expression in mucosal DCs and mesenteric lymph node (MLN) stromal cells. We subsequently showed that RA can directly regulate RALDH expression in DCs and stromal cells and we propose that intestinal epithelial cells are most likely the providers of RA for DCs. And thus, these intestinal epithelial cells provide an instructive environment that licenses DCs to transfer information about their tissue of origin and their site of antigen capture to the T cells. In addition, these imprinted DCs are crucial for other aspects of the mucosal immune system like the induction of regulatory T cells and IgA-producing B cells.

Developing immune system

Not only is dietary vitamin A of importance for the mucosal immune system, we also showed that it is affecting the formation of secondary lymphoid organs during ontogeny. We showed that RA was involved in embryonic lymph node development by promoting differentiation of lymphoid tissue inducer (LTi) cells. RA-supplemented mothers displayed increased numbers of mature LTi cells. Consistently, deprival of vitamin A led to a significant decrease of LTi differentiation status in developing LN of the embryos of vitamin A deficient (VAD) mothers.

Different levels of vitamin A converting RALDH enzymes

Differences in vitamin A metabolism will most likely result in altered functioning of the mucosal immune system. Inbred mouse strains show differences in RALDH activity. In the BALB/c mice, showing a higher RALDH activity when compared to C57BL/6 mice, a correlation was found between increased RALDH activity and enhanced induction of gut-homing molecule expression on $CD4^+$ T cells, higher FoxP3 levels in intestines and MLNs, increased IgA production, enhanced LTi differentiation, and larger MLNs and Peyers' patches (PPs). Moreover, in a disease setting, BALB/c mice showed an increased capacity to form tertiary lymphoid tissue that could contribute to IgA production and increased differentiation of regulatory T cells. Taken together, these data demonstrate that vitamin A is

of vital importance for proper functioning of the mucosal immune system and vitamin A status might be a determinant in disease susceptibility and severity.

Paracrine retinoic acid-mediated signalling

We have suggested that during embryonic lymph node development expression of CXCL13 by mesenchymal stromal organizer cells is induced by RA provided by adjacent nerves [1]. Also, many other examples of a paracrine function for RA come from studies regarding embryonic organogenesis (reviewed in [2]), while no genetic support for autocrine RA signalling exists. In addition, increasing numbers of examples of a paracrine mode of action for RA are described in the adult mouse. Intestinal epithelial cells are now thought to be the providers of RA during the differentiation and imprinting of mucosal DCs [3-5]. Moreover, we (chapter 2 and [6]) and others [7-10] have shown that the induction of gut-homing T cells depends on RA presented by DCs and MLN stromal cells. Since DCs are in close contact with T cells during T cell activation for presentation of antigen, we hypothesized that RA is transferred to and presented by DCs to T cells through the immunological synapse formed between T cells and DCs. Compatible with this idea are the reported observations that uptake and presentation of RA to T cells can occur by DCs upon pre-incubation with RA which did not involve newly formed RA [11]. However, it remains unknown how RA is secreted and transferred from one cell to another.

For transfer from DCs to T cells RA will have to be transported over both cell membranes. Molecules described to be especially equipped to perform such a function are ATP-binding cassette (ABC) transporters. ABC transporters are known to be expressed by a variety of immune cells like macrophages, DCs and T cells [12-17] and are thought to transport a wide variety of substrates across cellular membranes, including inflammatory molecules such as cytokines as well as lipids and drugs. Since RA is a lipid-soluble vitamin metabolite, ABC transporters might be good candidates for RA secretion. Indeed, overexpression of the ABC transporter MRP1 has been shown to block neuritogenic effects of RA in a neuroblastoma cell line, showing that MRP1 might have acted as an efflux pump for RA [18]. In addition, RA has been shown to regulate the expression of multiple ABC transporters, like P-glycoprotein (P-gp) [19, 20] or ABCA1 [21]. In this way, RA might regulate its own secretion. Similarly, expression of ABC transporters on T cells might be necessary for uptake of RA into the cell. Future research is needed to identify whether ABC transporters are involved in secretion and presentation of RA to neighboring T cells. Increasing the expression of ABC transporters on DCs, thereby enhancing RA secretion and presentation, may be beneficial to enhance the mucosal immune response necessary to overcome intestinal inflammation.

Modification of retinoic acid levels in the small intestine

Since RALDH expression in DCs is crucial for the mucosal immune system, it is of importance that we understand how RALDH levels in DCs are controlled. We have demonstrated in chapter 4 that after birth RALDH enzyme levels are upregulated in MLN-DCs and MLN stromal cells. This suggested to us that external factors are involved in inducing RALDH expression by these cells. Soon after birth, mucosal surfaces are colonized with high numbers of bacteria [22, 23], that play a crucial role in the maturation of the mucosal immune system [24-26]. Also, during the first week of life pups are solely fed with maternal milk, which is rich in vitamin A. In chapter 4, the role of these factors in regulating RALDH levels in mucosal DCs was investigated. We discovered that Toll-like receptor signalling was not crucial for RALDH enzyme expression since MLN-DCs from both MyD88$^{-/-}$ and Trif mutant animals had comparable levels of RALDH activity to WT animals, confirming the recent findings that MLN-DCs from MyD88$^{-/-}$ Trif$^{-/-}$ double knock-out mice still showed RALDH activity [27]. Remarkably, the intake of dietary vitamin A turned out to be mandatory for RALDH expression and enzyme activity within the mucosal immune system. Surprisingly, while RALDH mRNA levels in both MLN-DCs and MLN stromal cells were affected by the lack of vitamin A absorption, RALDH expression levels within the small intestine remained unchanged. This indicates that RALDH expression in mucosal DCs and intestinal epithelial cells is regulated differently. However, factors regulating RALDH levels within the intestinal epithelial cells are still unidentified. Since we did not observe an effect of the absence of MyD88- or Trif-dependent TLR signalling on epithelial-mediated imprinting of RALDH expression in mucosal DCs, we suspect that also in epithelial cells RALDH levels are not regulated by TLR-dependent signals. It is plausible that other pattern recognition receptor pathways or that secreted bacterial products are involved in regulation of RALDH enzymes in intestinal epithelial cells.

Being able to modify RALDH levels might be a beneficial tool to enhance RA production in the intestines, thereby promoting epithelial barrier function [28, 29], mucosal DC imprinting and mucosal tolerance induction. Moreover, adjusting vitamin A levels and RA production in pregnant mothers will likely have a significant effect on the development of the mucosal immune system of newborns. In chapter 7, we demonstrated that RA is involved in local differentiation of LTi cells during LN development. Increased intake of RA by pregnant mice correlated with higher numbers of mature LTi cells in the developing embryos, while LTi differentiation in developing embryos was reduced when vitamin A was omitted from the diet of the mothers. Because the numbers of LTi cells is associated with size and number of LNs ([30] and chapter 7), we expect that increased RA levels in pregnant mothers will promote the development of larger LNs in newborns. Additionally, after birth, LTi cells are thought to give rise to NKp46 mucosal cells, a cell type that has recently been described to enhance the antimicrobial response and epithelial barrier function by inducing anti-bacterial peptide production by

intestinal epithelial cells [31-33]. Modification of RA levels in pregnant mothers might therefore be beneficial to improve the mucosal immune system of their children.

Clinical implications

To demonstrate the significance of having increased RA-mediated signalling within the intestines, BALB/c and C57BL/6 mice were compared in chapter 5 and 6. These two mouse strains differ in RALDH activity and RA production in MLNs and small intestines. From these studies we can learn that increased RA-mediated signalling affected the mucosal immune system at multiple levels. The induction of gut-homing molecules and differentiation of regulatory T cells was enhanced in MLNs from BALB/c mice when compared to C57BL/6 mice. In addition, BALB/c mice displayed the accumulation of more T cells, FoxP3$^+$ Treg cells and IgA secreting B cells in the lamina propria of the small intestine. In addition, the presence of more mature LTi cells in BALB/c mice correlated with the formation of more secondary lymphoid tissue and the development of tertiary lymphoid structures during colitis when compared to C57BL/6. Taken together, enhanced RA production, and concomitant RA-mediated signalling, is associated with a more efficient mucosal immune system in BALB/c mice.

Cellular RA availability is regulated by the vitamin A nutritional status and the tightly regulated balance between RA synthesis and catabolism. Genetic variation (polymorphisms, SNPs and mutations) has been reported for a number of factors involved in maintaining this balance [34-39] like Cyp26A1 [37] or RALDH2 [36] and this may result in higher or lower RA synthesis. Variation in RA levels caused by mutations or polymorphisms in the genes encoding these factors in humans might determine the efficacy of the mucosal immune system and susceptibility for infectious disease. Possibly, factors like these are differentially expressed in BALB/c and C57BL/6, like RALDH enzymes shown in chapter 5 and 6. Consequently, BALB/c may have a better defense against mucosal infection and this might explain why these mice suffer from less severe colitis and recover more quickly than C57BL/6 mice.

This raises the attractive hypothesis that administration of RA to patients could serve as an effective treatment or alleviation of disease. A number of research groups have investigated the effect of RA treatment on several inflammatory diseases like colitis, experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), psoriasis and acne [40-47]. Indeed, RA downregulated inflammatory responses in colon biopsies cultured from patients with ulcerative colitis *in vitro* and in mice suffering from colitis *in vivo* by increasing the number of regulatory T cells [40]. EAE, an experimental model for multiple sclerosis, is an example of a typical Th17-driven immune disease in which RA was shown to strongly inhibit pathogenic Th17 responses during the disease. Treatment with RA

dramatically delayed disease onset but also strongly decreased disease severity [41, 42, 44, 45]. Also, RA treatment markedly ameliorated CIA, an experimental model for rheumatoid arthritis, in mice [43]. Disease onset was delayed and RA improved the clinical course by reducing the production of inflammatory cytokines, immunoglobulin, and chemokines. Taken together, these results support the hypothesis that RA could serve as a new therapeutic agent in the treatment of human inflammatory diseases.

Notably, the modification of RA availability must be approached with great carefulness. Because of the Th1-inhibiting effect of RA, Th1-driven immune responses like *Leishmania major* infection might be impaired. Also, excessive levels of RA may lead to disturbed embryonic development and birth defects. Recently involvement of RA in allergic responses has been described, where it is suggested that vitamin A deficiency or a high vitamin A intake may contribute to a lower or higher prevalence of allergic diseases, respectively [48-50]. In addition, as discussed in the introduction of this thesis, typical symptoms that are associated with high RA levels are weight loss, osteoporosis, liver damage, kidney damage, or loss of hair [51-57]. Therefore, when using RA as a therapeutic agent in patients, careful monitoring of adverse reactions must be pursued. Perhaps a more biological approach would be to modulate the levels of vitamin A converting RALDH enzymes within the intestines, since this may result in a more specific regulation of the amount of RA available to regulate the mucosal immune system. Future studies are needed to investigate how enzyme expression in the intestines can be modulated.

Concluding remarks

Based on the data presented in this thesis, we can conclude that vitamin A is of vital importance for mucosal immune homeostasis and vitamin A status in man might determine disease susceptibility and severity. In conclusion, the beneficial effect of RA on inflammatory disease in experimental models raises the attractive hypothesis that administration of RA to or enhanced intestinal RALDH expression in patients could serve as an effective treatment of inflammatory disease.

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8

9

Nederlandse samenvatting

Dankwoord

Curriculum vitae

List of publications

Index

Nederlandse samenvatting

Het mucosale immuunsysteem

De taak van ons afweersysteem is om ziekmakende bacteriën en virussen die ons lichaam binnendringen, op te ruimen en te verwijderen. In ons maag-darmstelsel wordt dit principe echter veel subtieler toegepast. Hierin leven miljoenen bacteriën, de darmflora. Veel van deze bacteriën zijn niet ziekmakend, maar juist gunstig omdat deze bacteriën noodzakelijk zijn voor de vertering van voedsel dat ons eigen lichaam niet kan afbreken. Echter, wanneer schadelijke bacteriën binnenkomen, kunnen zij infecties veroorzaken. Een goed functionerend afweersysteem in het maag-darmstelstel (vanaf nu het mucosale afweersysteem genoemd) zal dus tegen schadelijke bacteriën in onze darm een afweerreactie opwekken om zo het lichaam te beschermen tegen infecties en ziekten, maar zal tegelijkertijd tolerant zijn voor de onmisbare bacteriën die daar continu aanwezig zijn.
Het mechanisme waarmee ons afweersysteem onderscheid maakt tussen schadelijke en onschadelijke bacteriën is nog niet geheel duidelijk. Toch is het wel duidelijk dat een goede balans van het afweersysteem in de darmen zeer belangrijk is voor onze gezondheid, omdat verstoring van deze balans kan resulteren in chronische darmontstekingen zoals ulceratieve colitis en de ziekte van Crohn. Een beter begrip van deze balans is van groot belang voor de ontwikkeling van nieuwe therapieën voor de behandeling van dit soort ziekten.

Vitamine A is zeer belangrijk voor onze gezondheid. Het is essentieel voor veel processen zoals de embryonale ontwikkeling, voortplanting, groei, zicht en het mucosale afweersysteem. Ontoereikende consumptie van voedsel met vitamine A in ontwikkelingslanden resulteert in vitamine A deficiëntie dat voornamelijk voorkomt bij kinderen en zwangere vrouwen. Vitamine A deficiëntie leidt tot o.a. blindheid en groeistoornissen. Vitamine A deficiëntie ook tot een verslechterd afweersysteem waardoor diarree en darmontstekingen verhoogd en in ernstigere vorm voorkomen. Hierdoor overlijden jaarlijks meer dan een miljoen kinderen. Om vitamine A deficiëntie te voorkomen en te verhelpen heeft de Wereldgezondheidsraad programma's opgezet om kinderen in ontwikkelingslanden twee tot drie keer per jaar vitamine A toe te dienen. Dit heeft tot gevolg gehad dat kindersterfte is gedaald met 20-50%.
Vitamine A kan niet door ons lichaam geproduceerd worden en is een micronutriënt dat in de darmen wordt geabsorbeerd uit groente, fruit (wortels, pompoen, spinazie, boerenkool), dierlijk voedsel (lever, eieren) en zuivel. Vitamine A wordt in het lichaam omgezet tot retinolzuur. Retinolzuur is de werkzame stof die de hierboven beschreven processen beinvloeden. Voor de omzetting van vitamine A naar retinolzuur zijn enzymen nodig. Deze enzymen worden retinal dehydrogenase (afgekort RALDH) genoemd.
Dendritische cellen zijn belangrijke cellen van het afweersysteem die in de huid, longen en darmen continu op zoek zijn naar ziekteverwekkers waartegen ons immuunsysteem moet optreden. Wanneer deze zijn waargenomen, dan wordt de

informatie over de locatie en type gevaar door dendritische cellen gepresenteerd aan andere cellen van het immuunsysteem die de specifieke afweer moeten leveren. Dit zijn de T- en B-lymfocyten. De plek waar dit met grote efficiëntie gebeurd zijn de lymfoïde organen zoals de lymfeklieren en de milt. Wanneer lymfocyten in deze lymfoide organen de eiwitten afkomstig van de ziekteverwekkers kunnen herkennen, raken ze geactiveerd, waarna ze in staat zijn om tegen de ziekteverwekkers op te treden.
Wanneer lymfocyten in de darm drainerende lymfeklieren worden geactiveerd krijgen ze vervolgens specifieke eiwitten op hun cel oppervlak, waarmee ze uitermate goed naar de darmen kunnen migreren. Dit is belangrijk omdat hier de ziekteverwekkers zijn binnengedrongen en vervolgens onschadelijk gemaakt moeten worden. Wanneer ziekteverwekkers via de huid binnendringen, dan gaan de lymfocyten die geactiveerd worden in de huid drainerende lymfeklieren juist moleculen tot expressie brengen waardoor ze naar de huid kunnen migreren.
Vitamine A, in de vorm van de biologisch actieve metaboliet retinolzuur, reguleert de expressie van de moleculen die lymfocyten nodig hebben om naar de darm te migreren, de zogenaamde darmspecifieke homingsreceptoren. De benodigde RALDH enzymen voor de synthese van retinolzuur uit vitamine A worden specifiek door dendritische cellen van de darmen en in de darm drainerende lymfeklieren (mucosale dendritische cellen) tot expressie gebracht, terwijl de dendritische cellen buiten de darm en andere mucosa niet beschikken over deze enzymen.
Naast de inductie van darmspecifieke homingsreceptoren is retinolzuur ook belangrijk voor de productie van het type A antilichamen (IgA). Antilichamen worden door B lymfocyten geproduceerd en IgA wordt voornamelijk in mucosale weefsels, zoals de darm, geproduceerd om bescherming te bieden tegen de bacteriën die aanwezig zijn in de darm. Retinolzuur is zeer belangrijk voor de productie van IgA door B cellen.

Kort samengevat, vitamine A is zeer belangrijk voor het functioneren van het mucosale afweersysteem, omdat verschillende immunologische processen beïnvloedt worden door de biologische werkzame metaboliet retinolzuur. Toch is er nog veel onbekend over de specifieke mechanismes waarmee vitamine A de gezondheid en het afweersysteem bevorderd en de ontwikkeling van infecties verlaagd.
In dit proefschrift worden studies beschreven waarin we de rol van vitamine A in verschillende onderdelen van het afweersysteem hebben onderzocht. Onze resultaten dragen bij aan een beter inzicht in de beschermende rol van vitamine A in het mucosale immuunsysteem.

Samenvatting van het proefschrift

In **hoofdstuk 2** laten wij zien dat naast mucosale dendritische cellen ook stromale cellen (of steuncellen) in mesenteriale lymfeklieren RALDH enzymen produceren. Dit betekent dat stromale cellen ook retinolzuur kunnen produceren en T en B lymfocyten kunnen beïnvloeden. Om dit te onderzoeken hebben we mesenteriale

lymfeklieren getransplanteerd naar een gebied buiten de mucosa, namelijk de knieholte van de muis. Vervolgens hebben we onderzocht of T cellen in deze lymfeklieren worden gestimuleerd om darmspecifieke eiwitten tot expressie te brengen. De lymfeklier in de knieholte draineert de huid en en hier worden normaal gesproken alleen de huidspecifieke homingsreceptoren geinduceerd op geactiveerde lymfocyten. Na transplantatie verdwijnen de afweercellen uit de getransplanteerde lymfeklier verdwijnen en hiermee dus ook de mucosale RALDH-positieve dendritische cellen. De stromale cellen blijven achter en zijn dan het enige celtype verantwoordelijk voor de productie van retinolzuur. Wanneer lymfocyten in de getransplanteerde mesenteriale lymfeklier worden geactiveerd dan resulteert dit in de expressie van een deel van de darmspecifieke homingsreceptoren, terwijl een ander deel afhankelijk bleek te zijn van de mucosale dendritische cellen. Onze experimenten tonen dus aan dat dendritische cellen en stromale cellen in mesenteriale lymfeklieren beide T cellen kunnen instrueren om darmspecificieke eiwitten tot expressie te brengen en om zo efficient mogelijk T cellen naar de juiste weefsels te sturen.

In **hoofdstuk 3** hebben we onderzocht hoe darmepitheelcellen de ontwikkeling van mucosale DCs beïnvloeden. We hebben laten zien dat de darmepitheel cellen de dendritische cellen aanzetten om de vitamine A metaboliserende enzymen RALDH tot expressie te brengen. Deze dendritische cellen stimuleerden vervolgens T cellen om de darm-specifieke homingsreceptoren tot expressie te brengen. Retinolzuur, afkomstig uit de darm epitheel cellen, bleek in dit proces een cruciale factor te zijn. Daarentegen werden dendritische cellen door steuncellen uit de huid juist zo beinvloed, dat zij lymfocyten stimuleerden om de huid-specifieke homingsreceptoren tot expressie te brengen.

In **hoofdstuk 4** hebben we onderzocht hoe de expressie van RALDH enzymen in dendritische cellen en stromale cellen wordt gereguleerd. Onze resultaten lieten zien dat vlak na de geboorte zowel dendritische cellen als stromale cellen zeer lage expressie van RALDH enzymen vertoonden en dat tijdens de ontwikkeling na de geboorte de expressieniveaus in beiden celtypen verder omhoog gingen.
Parallel aan de verhoging van de RALDH expressie vindt na de geboorte ook de bacteriële kolonisatie van de darmen plaats, waarbij allerlei immunologische veranderingen in de darm plaatsvinden. Daarom hebben wij onderzocht of er een verband is tussen bacteriële activatie van dendritische cellen en de inductie van RALDH enzymen. Stimulatie van dendritische cellen in kweekschaaltjes met verschillende bacteriële componenten leidde in een aantal gevallen inderdaad tot het aanzetten van RALDH expressie in de dendritische cellen. Echter, in een levende muis (*in vivo*) lijkt bacteriële signalering minder belangrijk voor de inductie van RALDH enzymen. Dit bleek uit experimenten met mucosale dendritische cellen uit transgene muizen waarin bacteriële signalering is platgelegd. Hierdoor worden de dendritische cellen niet door bacteriën geactiveerd, maar deze vertoonden desondanks nog steeds actieve RALDH enzymen.
Omdat we in hoofdstuk 3 hadden gevonden dat epitheelcellen dendritische

cellen kunnen stimuleren om RALDH tot expressie te brengen en dat dit proces afhankelijk was van retinolzuur, hebben wij vervolgens onderzocht of vitamine A de RALDH expressie in dendritische cellen kan beïnvloeden. Om dit te onderzoeken hebben we vitamine A deficiënte muizen gegenereerd door muizen op een vitamine A-vrij dieet te zetten. Als controle werden ook muizen op diëten met verschillende hoeveelheden vitamine A gegenereerd. Tot onze verbazing vertoonden de mucosale dendritische cellen uit de vitamine A deficiënte muizen helemaal geen RALDH activiteit meer, terwijl deze cellen uit de controle dieren wel beschikten over actieve RALDH enzymen. Dit fenomeen was te herstellen door de vitamine A deficiënte dieren voor een periode van 7 dagen op vitamine A controle voer te zetten. Deze resultaten laten zien dat de expressie van RALDH enzymen in mucosale dendritische cellen volledig afhankelijk is van de aanwezigheid van vitamine A in het voer.

In **hoofdstuk 5** hebben we het vitamine A metabolisme van twee verschillende muizenstammen vergeleken, namelijk BALB/c en C57BL/6 muizen. De BALB/c muizen hadden hogere expressie niveaus van RALDH enzymen in zowel darmen als mesenteriale lymfeklieren. Hierdoor zijn BALB/c muizen in staat zijn om meer retinolzuur te produceren in de darmen en de mesenteriale lymfeklieren dan C57BL/6 muizen. Als gevolg hiervan is de inductie van de darmspecifieke homingsreceptoren hoger in BALB/c muizen in vergelijking met C57BL/6 muizen. De consequentie hiervan is dat er meer lymfocyten aanwezig zijn in de darmen van BALB/c muizen in vergelijking met C57BL/6 muizen Door de verhoogde retinolzuur productie lijken BALB/c muizen dus te beschikken over een beter functionerend mucosaal afweersysteem dan C57BL/6 muizen.

Dit laatste blijkt ook uit onze colitis studie, welke is beschreven in **hoofdstuk 6**. Hierin hebben we de BALB/c muizen en C57BL/6 muizen opnieuw met elkaar vergeleken in een model waarin colitis werd geinduceerd. Colitis is een chronische ontsteking van de darmen en wordt in onze muismodellen geinduceerd door aan drinkwater het stofje dextraan sulfaat sodium (DSS) toe te voegen, waardoor een beschadiging van het darm epitheel ontstaat, welke leidt tot een tijdelijke ontsteking. BALB/c en C57BL/6 muizen verschilden in gevoeligheid voor de ontwikkeling van deze ziekte. Het bleek dat BALB/c muizen een hogere dosis van DSS nodig hadden om een vergelijkbaar ziektebeeld te ontwikkelen als C57BL/6 muizen. Daarnaast herstelden BALB/c muizen sneller van colitis wanneer DSS uit het drinkwater werd verwijderd, zelfs na toediening van de hoge dosis DSS. We toonden aan dat BALB/c muizen tijdens colitis meer lymfoide structuren in de dikke darm ontwikkelden dan C57BL/6 muizen en dat deze lymfoide structuren veel IgA producerende B lymfocyten en en beschermende T lymfocyten bevatten. Om te bewijzen dat vitamine A daadwerkelijk belangrijk is voor bescherming tegen colitis hebben we colitis geïnduceerd in vitamine A deficiënte dieren en deze vergeleken met vitamine A controle dieren. Beide groepen ontwikkelden colitis, echter het ziektebeeld in de vitamine A deficiënte dieren was vele malen slechter dan in de vitamine A controle dieren. Deze resultaten tonen aan dat vitamine A een

beschermende invloed heeft op colitis en dat een hogere retinolzuurproductie, zoals in BALB/c muizen, correleert met een lagere gevoeligheid voor de ontwikkeling van colitis en dus bijdraagt aan een beter functionerend mucosaal afweersysteem.

In **hoofdstuk 7** van mijn proefschrift wordt besproken hoe retinolzuur de embryonale ontwikkeling van lymfeklieren beïnvloedt. Voor de embryonale ontwikkeling van lymfeklieren zijn lymphoid tissue inducer (LTi) cellen en stromale organizer cellen nodig. Wij toonden *in vitro* aan dat de differentiatie van precursor LTi cellen tot volledig uitgerijpte LTi cellen werd bevorderd door de aanwezigheid van retinolzuur. Wanneer retinolzuur werd toegediend aan het voer van zwangere muizen leidde dit tot de ontwikkeling van meer uitgerijpte LTi cellen in het embryo. Echter, wanneer een zwangere muis op vitamine A deficiënt voer werd gezet, nam het aantal uitgerijpte LTi cellen in het embryo af. Ook in de BALB/c muizen die meer retinolzuur kunnen produceren waren meer uitgerijpte LTi cellen in de embryo's aanwezig dan in C57BL/6 embryo's. Het aantal uitgerijpte LTi cellen correleerde met de grootte van lymfeklieren na de geboorte. Uit deze analyses komt zeer duidelijk naar voren dat BALB/c muizen meer lymfoide structuren in de darmen hebben en grotere Peyerse platen en mesenteriale lymfeklieren hebben dan C57BL/6 muizen. Dit impliceert dat de vitamine A status (retinolzuurproductie en bloedspiegel) van de moeder invloed kan hebben op de ontwikkeling van het mucosale afweersysteem van haar kind.

Conclusie

De studies beschreven in dit proefschrift laten zien dat vitamine A zeer belangrijk is voor het functioneren van ons mucosale afweersysteem vanwege de positieve invloed van het vitamine A metaboliet retinolzuur op verschillende onderdelen van het mucosale afweersysteem. Onvoldoende inname van vitamine A kan leiden tot een verstoorde balans van het mucosale afweersysteem wat de ontwikkeling van darmontstekingen en infecties tot gevolg kan hebben. Dit impliceert dat de effectiviteit van het mucosale afweersysteem en de gevoeligheid voor darminfecties en ontstekingen in mensen mede bepaald wordt door de inname en omzetting van vitamine A. Daarom kan de toediening van retinolzuur of mogelijkheid om RALDH niveaus te reguleren in patiënten een effectieve methode zijn om retinolzuur niveaus in de darmen te verhogen en zo het mucosale afweersysteem te versterken tijdens de behandeling van chronische darmontstekingen zoals ulceratieve colitis of de ziekte van Crohn.

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Rosalie

Curriculum Vitae

Rosalie Molenaar werd op 1 december 1981 geboren te Alkmaar. Zij behaalde haar VWO diploma in 2000 aan het Petrus Canisius College te Alkmaar, waarna zij in september van dat jaar begon met de opleiding Biomedische Wetenschappen aan de Vrije Universiteit te Amsterdam. Tijdens deze opleiding verrichte zij haar eerste wetenschappelijke stage op de afdeling Moleculaire Celbiologie en Immunologie van het VU Medisch Centrum te Amsterdam onder begeleiding van dr. Janneke Samsom, waar zij heeft meegewerkt aan het onderzoek naar regulatoire T cellen. Tijdens de master-fase van de opleiding Biomedische Wetenschappen heeft Rosalie zich gespecialiseerd in de immunologie. Tijdens deze fase heeft zij als stagiaire onderzoek gedaan naar de functie en detectie van granzyme B en proteinase inhibitor 9 in serum tijdens transplantatie afstotingsreacties in nierpatienten. Dit onderzoek werd uitgevoerd op de afdeling Experimentele Immunologie op het Academisch Medisch Centrum te Amsterdam onder supervisie van prof.dr. Ineke ten Berge en dr. Ajda Rowshani en op de afdeling Plasma Eiwitten van Sanquin Research onder begeleiding van prof. dr. Erik Hack. De afrondende stage heeft Rosalie gedaan op het Nederlands Kanker Instituut op de afdeling Moleculaire Genetica, onder supervisie van dr. Jacqueline Jacobs en prof.dr. Anton Berns, waar onderzoek werd verricht naar factoren die de telomeren van chromosomen beschermen. In september 2005 heeft Rosalie haar master diploma Biomedische Wetenschappen behaald en is zij vervolgens in december van hetzelfde jaar gestart met haar promotieonderzoek bij de afdeling Moleculaire Celbiologie en Immunologie van het VU Medisch Centrum te Amsterdam onder begeleiding van promoteren prof.dr. R.E. Mebius en prof.dr. G. Kraal. De resultaten van dit onderzoek staan beschreven in dit proefschrift met de titel: Control of mucosal immune responses by vitamin A. Sinds 1 april 2010 is Rosalie als post-doc werkzaam op de afdeling Moleculaire Celbiologie en Immunologie van het VU Medisch Centrum te Amsterdam om het onderzoek wat is opgezet tijdens haar promotie voort te zetten.

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