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Expression of Retinaldehyde Dehydrogenase Enzymes in Mucosal Dendritic Cells and Gut-Draining Lymph Node Stromal Cells Is Controlled by Dietary Vitamin A

Rosalie Molenaar,* Marlene Knippenberg,* Gera Goverse,* Brenda J. Olivier,* Alex F. de Vos, † Tom O’Toole,* and Reina E. Mebius*

The vitamin A metabolite retinoic acid (RA) plays a crucial role in mucosal immune responses. We demonstrate in this study that RA-producing retinaldehyde dehydrogenase (RALDH) enzymes are postnatally induced in mesenteric lymph node (MLN) dendritic cells (DCs) and MLN stromal cells. RALDH enzyme activity in lamina propria-derived CD103+ MLN-DCs did not depend on TLR signaling. Remarkably, RA itself could directly induce RALDH2 in both DCs and stromal cells in vitro. Furthermore, upon provision of a vitamin A-deficient diet, it was found that RA-mediated signaling was strongly reduced within the small intestines, while RALDH2 mRNA and RALDH enzyme activity in lamina propria DCs and MLN-DCs, as well as RALDH2 mRNA expression in MLN stromal cells, were strongly diminished. Moreover, supply of vitamin A to vitamin A-deficient mice restored RA-mediated signaling in the intestine and RALDH activity in lamina propria-derived CD103+ MLN-DCs. Our results show that RA-dependent signaling within the intestine is indispensable for RALDH activity in the draining MLN.

Vitamin A has long been recognized for its role in immunity. It is currently estimated that 190 million school-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 (ALDH1A1, also called retinaldehyde dehydrogenase [RALDH]) enzymes, dendritic cells (DCs) and stromal cells in gut-associated lymphoid organs, such as Peyer’s patches and mesenteric lymph nodes (MLNs), 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 cells (12–18), and IgA-producing B cells (19–21), as well as to suppress the differentiation of Th17 cells (13, 16, 17). In the absence of dietary vitamin A, gut-homing of T cells and B cells to the intestines is disturbed (7, 20). 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 RA levels are regulated within the mucosal immune system.

Vitamin A is first reversibly oxidized by alcohol dehydrogenases 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, such as intestinal epithelial cells (7, 25–27), nerve fibers (28), and MLN stromal cells as well as DCs in Peyer’s patches, 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 and stromal cells 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 these cells 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 RA receptor (RAR) signaling in T cells (29), Foxp3 regulatory T cell differentiation, and gut-homing receptor expression on activated T cells when compared with 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 DCs (BMDCs) with gut epithelial cells induced expression of RALDH enzymes and educated BMDCs 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, TGF-β, thymic stromal...
lymphopoietin, 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). Whether these cytokines also affect RALDH expression in lymph node (LN) stromal cells has not been addressed.

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 TLRs (38, 39), C-type lectin receptors (40), and intracellular Nod-like receptors (41). Recently, it was described that RALDH2 expression in both BMDCs 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.

In this study, 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 are independent of TLR signaling, because MLN-DCs from Trif mutant, MyD88−/−, and C57BL/6 wild-type animals exhibit similar RALDH activity. Remarkably, dietary vitamin A appeared to be crucial for RALDH expression in mucosal DCs and MLN stromal cells, and loss of RALDH activity in MLN-DCs from mice that were fed a vitamin A-deficient diet could quickly be 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 1–14 wk, MyD88−/− mice (43), and Trif−/− mice aged 10–14 wk were bred at our own animal facilities. All mice were housed under specific pathogen-free conditions. In Trif−/− mice, the Trif protein is modified by a single base pair deletion, resulting in a dysfunctional protein (44). The Animal Experiments Committee of the Vrije University 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 Laboratories (Maastricht, The Netherlands) were mated and pregnant females either received a chemically defined diet that lacked vitamin A (the modified AIN-93M feed; MP Biomedicals, Solon, OH), or vitamin A-deficient diet (VAD) containing vitamin A (200 IU/kg in the modified AIN-93M feed; MP Biomedicals; vitamin A control (VAC), or contained double the amount of vitamin A (5600 IU/kg in the modified AIN-93M feed; MP Biomedicals; vitamin A high (VAH)). All mice were housed under specific pathogen-free conditions. These diets started at 8–9 d of gestation. The pups were weaned at 4 wk of age and maintained on the same diet at least until 10 wk of age before analysis was performed.

Preparation of small intestine cell and LN suspensions

Small intestines were dissected and opened longitudinally after removal of mesenteric fat. Small intestines were cut 15 mm HEPES (Invitrogen) and 250 μg/ml gentamicin to remove fecal contents. Small intestines were washed with HBSS without Ca²⁺ and Mg²⁺ (Invitrogen) to discriminate between live versus dead cells. Secondary Ab used for staining kits (StemCell Technologies), according to the manufacturer’s protocol with modifications. Briefly, cells suspended at 10⁶ cells/ml in ALDEFLUOR assay buffer containing activated ALDEFLUOR substrate (365 nM) with or without the RALDH inhibitor diethylaminobenzaldehyde-hyde (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) or Alexa eFlour 450-conjugated anti-CD11c (clone N418; eBioscience), biotin-conjugated anti-CD103 (clone M290; BD Biosciences), Allexa Fluor 647-conjugated anti–MHC-II (clone M5/114), and with Sytox Blue (Invitrogen) to discriminate between live versus dead cells. Secondary Ab used was PerCP-conjugated (BD Biosciences), PE-Cy7–conjugated streptavidin (eBioscience), or allophycocyanin-conjugated streptavidin (eBioscience). Cells were analyzed with a Cyan ADP flow cytometer (Beckman Coulter).

In vitro experiments

IMDM medium (Life Technologies, Grand Island, NY) containing 10% FCS (HyClone Laboratories/Greiner Bio-One), 50 μM 2-ME (Merck, Darmstadt, Germany), 1% l-glutamine, and 1% penicillin-streptomycin (BioWhittaker Europe, Verviers, Belgium) was used.

Bone marrow was isolated from femurs and tibia using a mortar and cultured for 1 wk in IMDM medium (Life Technologies) containing 10% FCS (HyClone Laboratories, Logan, Utah), 50 μM 2-ME (Merck), 1% l-glutamine, 1% penicillin-streptomycin (BioWhittaker Europe; IMDM complete medium), and 20 ng/ml GM-CSF (clone X63) to obtain BMDCs. BMDCs (1 × 10⁶) were cultured in 96-well plates (Greiner Bio-One) for 2 wk in a volume of 200 μl IMDM complete medium in the presence or absence of 100 nM all-trans-RA (Sigma-Aldrich). Stromal cells from PLNs and MLNs were cultured in 96-well plates (Greiner Bio-One) for 7 d 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 h. Cells were lysed in RLT buffer from an RNeasy kit (Qiagen Benelux, Venlo, The Netherlands) or by 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-II⁺CD11c⁺ DCS, CD45⁺MHC-II⁺CD11c⁻ LN stromal cells, and stimulated BMDCs and LN stromal cells using a RNeasy kit (Qiagen Benelux) or miRNA Capture kit (Roche) according to the manufacturers’ protocols, and cDNA was synthesized using a RevertAid First Strand cDNA synthesis kit (Fermentas Life Sciences, Burlington, Ontario, Canada) or with a reverse transcription system (SuperScript III, Invitrogen). For RALDH1 (Aldh1a1), RALDH2 (Aldh1a2), RALDH3 (Aldh1a3), RARβ, and GM-CSF (Csf2) and for housekeeping genes ubiquitin C, hprt, and GAPDH (Isogen Life Science, De Meern, The 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 each primer, and SYBR Green Master Mix (PE Applied Biosystems).

Cell sorting, Aldefluor assay, and flow cytometry

For sorting of DCS and stromal cells, cells were stained with biotin-conjugated anti-MHC class II (MHC-II; clone M5/114), PE-conjugated anti-CD11c (clone N418; eBioscience), Alexa Fluor 647 (Invitrogen)-conjugated anti-CD45 (clone MP33, and 7-aminocinomycin D (Molecular Probes/Invitrogen) to discriminate between live versus dead cells. CD45⁺MHC-II⁺CD11c⁺ DCS and CD45⁺MHC-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), according to the manufacturer’s protocol with modifications. Briefly, cells suspended at 10⁶ cells/ml in ALDEFLUOR assay buffer containing activated ALDEFLUOR substrate (365 nM) with or without the RALDH inhibitor diethylaminobenzaldehyde-hyde (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) or Alexa eFlour 450-conjugated anti-CD11c (clone N418; eBioscience), biotin-conjugated anti-CD103 (clone M290; BD Biosciences), Alexa Fluor 647-conjugated anti–MHC-II (clone M5/114), and with Sytox Blue (Invitrogen) to discriminate between live versus dead cells. Secondary Ab used was PerCP-conjugated (BD Biosciences), PE-Cy7–conjugated streptavidin (eBioscience), or allophycocyanin-conjugated streptavidin (eBioscience). Cells were analyzed with a Cyan ADP flow cytometer (Beckman Coulter).

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Immunofluorescence stainings

Small intestines from VAD and VAC mice were embedded in OCT compound (Sakura Finetek Europe) and stored at 280˚C. Seven-micrometer cryosections were dehydrated in acetone for 10 min and air-dried for an additional 10 min. Sections were stained with anti–neuron-specific βIII-tubulin Ab (clone TUJ-1; Abcam), anti-Raldh1/2 (Abcam; catalog no. ab23375), and visualized with Alexa Fluor 488-labeled anti-mouse IgG2a (Invitrogen) and Alexa Fluor 555-labeled anti-rabbit IgG (Invitrogen), respectively. Sections were analyzed with the DM6000 Leica immunofluorescence microscope (Leica Microsystems, Rijswijk, The Netherlands) and Photoshop (CS3; Adobe).

Statistics

Results are given as the means ± SD. Statistical analyses were performed using a two-tailed Student t test.

Results

RALDH expression and activity are 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 molecules on T cells within adult MLNs. 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. Accordingly, MHC-II+ CD11c+ DCs and CD45+ MLN stromal cells were sorted from MLNs and PLNs from C57BL/6 mice before weaning (2–3 wk of age) and after weaning (7–9 wk 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, whereas adult MLN-DCs highly expressed RALDH2 (Fig. 1A). A similar age-related distribution was found for CD45+ MLN stromal cells sorted from mice at the age of 2–3 wk, which showed significantly lower expression of RALDH2 when compared with adult MLN stromal cells (Supplemental Fig. 1A). Notably, RALDH2 expression in PLN stromal cells was undetectable 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 increased postnatally during the course of 2–7 wk.

Low mRNA expression of RALDH2 in MLN-DCs at the age of 2–3 wk 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 wk after birth ~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⁺MHC-II⁺CD11c⁺ cells of MLNs (Fig. 1C, left), corroborating that CD103⁺ MLN-DCs have higher RALDH mRNA levels compared with CD103⁻ DCs in MLNs (12). Notably, we were not able to measure RALDH activity in MLN stromal cells with the ALDEFLUOR assay. However, we have shown that MLN stromal cells have functional RALDH enzymes since these cells can imprint gut-homing molecules on T cells directly (10). At 1 and 3 wk of age, CD103⁺ MLN-DCs already showed RALDH activity, which was significantly higher when compared with PLN-DCs at this age, unlike mRNA levels. However, at 2-3 wk of age, levels of RALDH activity in CD103⁺ MLN-DCs were significantly lower than measured in adult CD103⁺ MLN-DCs (Fig. 1A). Additionally, CD103⁺ PLN-DCs showed very low RALDH activity at all ages analyzed. In conclusion, not only RALDH mRNA levels, but also RALDH enzyme activity increased in MLN-DCs during postnatal development.

**RALDH enzyme activity is not dependent on TLR signaling**

Next, we questioned how the expression and activity of RALDH are 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. However, recently it was published that RALDH activity was only slightly diminished in MLN-DCs in germ-free mice as well as in mice with a combined deficiency for MyD88 and Trif, indicating that TLR signaling might not be involved in the induction of RALDH activity in MLN-DCs in vivo (47). Because it has been described that DCs can be induced to express RALDH2 upon TLR2 triggering in vitro (42), we wanted to ensure that RALDH enzyme activity in MLN-DCs was not dependent on TLR signaling in vivo. Analysis of MLN-DCs from MyD88⁻/⁻ (Fig. 2A) and Trif mutant mice (Fig. 2B) indeed showed that normal RALDH enzyme activity could be measured in these DCs, substantiating that TLR signaling via these adaptor proteins is not mandatory for induction of RALDH in MLN-DCs in vivo.

**RALDH enzyme activity is abrogated in vitamin A-deficient mice**

Because it has been shown that intestinal epithelial factors, among which is the vitamin A metabolite RA, induce RALDH expression in BMDCs (35), we decided to address whether dietary vitamin A is crucial for the expression and activity of RALDH enzymes in mucosal DCs. C57BL/6 mice were raised on a VAD diet and comparable to levels observed in PLN-DCs (Fig. 3C). In all three groups the same percentage of CD103⁺ DCs was present within the MLN (data not shown). Additionally, RALDH enzyme activity in MLN-DCs from VAD and VAH mice was comparable, indicating that double the amount of dietary vitamin A did not further increase RALDH enzyme activity in MLN-DCs (Fig. 3C). 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 mucosal DCs required dietary vitamin A, RALDH2 mRNA expression levels in MLN-DCs from VAD mice were significantly reduced when compared with MLN-DCs from VAC mice (Fig. 3D). Additionally, CD45⁺ MLN stromal cells sorted from VAD mice also showed significantly lower levels of RALDH2 mRNA when compared with VAC mice (Supplemental Fig. 1B). In conclusion, these data show that dietary vitamin A is required for RALDH enzyme activity in mucosal DCs and that the expression and activity of RALDH enzymes in lamina propria derived DCs are 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. However, recently it was published that RALDH activity was only slightly diminished in MLN-DCs in germ-free mice as well as in mice with a combined deficiency for MyD88 and Trif, indicating that TLR signaling might not be involved in the induction of RALDH activity in MLN-DCs in vivo (47). Because it has been described that DCs can be induced to express RALDH2 upon TLR2 triggering in vitro (42), we wanted to ensure that RALDH enzyme activity in MLN-DCs was not dependent on TLR signaling in vivo. Analysis of MLN-DCs from MyD88⁻/⁻ (Fig. 2A) and Trif mutant mice (Fig. 2B) indeed showed that normal RALDH enzyme activity could be measured in these DCs, substantiating that TLR signaling via these adaptor proteins is not mandatory for induction of RALDH in MLN-DCs in vivo.

Because it has been shown that intestinal epithelial factors, among which is the vitamin A metabolite RA, induce RALDH expression in BMDCs (35), we decided to address whether dietary vitamin A is crucial for the expression and activity of RALDH enzymes in mucosal DCs. C57BL/6 mice were raised on a VAD diet and compared with animals raised on a VAC or VAH diet, which contained double the amount of vitamin A. Serum retinol levels in 10-wk-old VAC and VAH mice were 1.30 ± 0.01 and 1.43 ± 0.17 μM, respectively, whereas serum retinol levels in VAD mice were undetectable at this age (detection limit 0.10 μM, Supplemental Fig. 2). Remarkably, the absence of dietary vitamin A resulted in a significant reduction of RALDH enzyme activity in CD103⁺ MHC-II⁺CD11c⁺ DCs in the lamina propria of the small intestine, as ALDEFLUOR levels measured in mucosal DCs from VAD mice were significantly lower when compared with mucosal DCs from VAC mice (Fig. 3A). Furthermore, RALDH2 mRNA expression levels in purified CD45⁺ lamina propria cells, containing mucosal DCs, from VAD mice were significantly reduced when compared with cells from VAC mice (Fig. 3B).

Consequently, ALDEFLUOR levels measured in lamina propria-derived CD103⁺ MLN-DCs from VAD mice were reduced by 94% when compared with MLN-DCs from VAC and VAH mice and comparable to levels observed in PLN-DCs (Fig. 3C). In all three groups the same percentage of CD103⁺ DCs was present within the MLN (data not shown). Additionally, RALDH enzyme activity in MLN-DCs from VAD and VAH mice was comparable, indicating that double the amount of dietary vitamin A did not further increase RALDH enzyme activity in MLN-DCs (Fig. 3C). 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 mucosal DCs required dietary vitamin A, RALDH2 mRNA expression levels in MLN-DCs from VAD mice were significantly reduced when compared with MLN-DCs from VAC mice (Fig. 3D). Additionally, CD45⁺ MLN stromal cells sorted from VAD mice also showed significantly lower levels of RALDH2 mRNA when compared with VAC mice (Supplemental Fig. 1B). In conclusion, these data show that dietary vitamin A is required for RALDH enzyme activity in mucosal DCs and that the expression and activity of RALDH enzymes in lamina propria derived DCs are regulated during postnatal development.
we could rescue this by feeding the VAD animals a VAH diet for 7 d (VAD > VAH). Indeed, supplementation with vitamin A resulted in a significant increase of Aldefluor+CD103+ DCs in MLNs of these mice when compared with VAD animals (Fig. 4A, 4B). Already after 7 d, the RALDH activity in MLN-DCs from supplemented animals was almost back to levels of RALDH activity as observed in VAH MLN-DCs. 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 (Fig. 4C). These results show that already after 7 d 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.

RA can directly regulate RALDH enzyme expression in LN stromal cells and DCs

Our data suggested that RA, derived from vitamin A, may be crucial for RALDH expression in mucosal DCs and MLN stromal cells, and we hypothesized that RA 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 could be induced by addition of increasing amounts of RA in vitro. PLN stromal cells, which originally display very low expression of RALDH2 mRNA (Fig. 5A, left, control), showed a significant induction of RALDH2 expression upon stimulation with 50 nM RA (Fig. 5A, left). However, at high RA concentrations (500

**FIGURE 3.** Dietary vitamin A regulates RALDH enzyme expression and activity in DCs of small intestine and in MLNs. A and C, CD103+MHC-II+C11cighb DCs in small intestines (A) and MLNs (C) from C57BL/6 mice on VAD and VAH diet aged 10 wk were analyzed for RALDH activity using the ALDEFLUOR assay. Five to seven animals per group were used for analysis. Experiment was performed twice. Graphs show ALDEFLUOR mean fluorescence intensity (MFI) ± SD in CD103+ DCs from VAD and VAH small intestines (A) and in CD103+ MLN-DCs and PLN-DCs from VAD, VAH, and VAH mice (C) in absence (black bars) or presence (gray bars) of RALDH inhibitor DEAB. B and D, CD45+ purified cells from small intestines (B) and CD45+C11cighb FACS-sorted MLN-DCs (D) from 11-wk-old VAD and VAH mice were analyzed for expression of RALDH1, RALDH2, and RALDH3 by real-time PCR. Relative RALDH2 expression levels in VAD CD45+ cells (B) and in VAH MLN-DCs (D) was set at 1 and were normalized to cyclophilin A and GAPDH. RALDH1 and RALDH3 expression were too low for reliable measurements. Five to six animals per group were used. *p < 0.05; **p < 0.01; ***p < 0.001.

**FIGURE 4.** RALDH activity in VAD MLN-DCs is restored upon vitamin A supplementation. MHC-II+C11cighb DCs in MLN cell suspensions from C57BL/6 mice, aged 12 wk, on VAD diet, VAH diet, or from VAD mice that received VAH diet for 7 d (VAD > VAH) were analyzed for CD103 expression and RALDH activity using an Aldefluor assay. A. Shown are representative flow cytometry plots of Aldefluor activity and CD103 expression in MLN suspensions from VAH, VAD, and VAH mice. Box indicates CD103 gate for calculations of Aldefluor mean fluorescence intensity (MFI) shown in B. B. Graphs show Aldefluor MFI ± SD in MLN-DCs from VAD, VAH, and VAH mice in absence (black bars) or presence (gray bars) of RALDH inhibitor DEAB. Eight animals per group were used for analysis. Experiment was performed twice. C, RNA was isolated from MLNs from VAH, VAD, and VAH mice and analyzed for RALDH2 expression with real-time PCR. Expression of transcripts was normalized to GAPDH. Three MLNs per group were used for analysis. RALDH1 and RALDH3 expression were undetectable in these samples. *p = 0.05; **p < 0.002.
nM) no induction of RALDH2 expression could be observed. Additionally, analysis of MLN stromal cells demonstrated that RALDH2 expression in these cells could not be further increased by addition of RA. Instead, a reduction of RALDH2 expression with increasing amounts of RA was observed in these cells (Fig. 5A, right). These data suggest that high concentrations of RA prevent expression of RALDH2 enzymes, most likely to prevent cellular RA toxicity. Incubation of stromal cells with the different concentrations of RA did not affect the viability of the cells upon analysis by microscopy.

Furthermore, BMDCs 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). Because 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 mucosal DCs. For induction of RALDH expression in MLN stromal cells, the transport of vitamin A by the lymph may be of additional 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**

Because 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 (Fig. 6A). RALDH3 expression was significantly increased during vitamin A deficiency, indicating the presence of a compensatory mechanism by increasing RALDH3 expression. We have previously observed that RALDH2 is also expressed in peripheral nerves (28), which may contribute here to the expression levels of RALDH2 measured in the small intestines. Indeed, stainings for RALDH showed that βIII-tubulin+ nerves within the intestines of both VAD and VAC mice expressed the enzyme (Supplemental Fig. 3).

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

**FIGURE 5.** Regulation of RALDH expression in DCs and LN stromal cells by RA. A, PLN and MLN stromal cells were cultured for 7 d and subsequently stimulated with 5, 50, or 500 nM RA. After 24 h, PLN stromal cells (A, left) and MLN stromal cells (A, right) were analyzed for RALDH2 expression with real-time PCR. B, BMDCs were cultured alone or in the presence of 100 nM RA. After 24 h, BMDCs were analyzed for expression of RALDH1, RALDH2, and RALDH3 by real-time PCR. Relative expression levels in unstimulated MLN stromal cells (control; A, right) and in control DCs (DC only; B) were set at 1 for each RALDH. Expression of transcripts was normalized to GAPDH. Experiments were performed three times. *p < 0.001; **p = 0.01; ***p = 0.05.

**FIGURE 6.** Expression of RALDH enzymes in small intestines is not affected by dietary vitamin A deficiency. A and B, Expression of RALDH1, RALDH2, and RALDH3 mRNA (A) and RARβ mRNA (B) were analyzed in small intestine samples from VAD and VAC mice by real time PCR. C, Expression levels of RARβ mRNA were analyzed in small intestine samples from VAD, VAH, and VAD mice supplemented for 7 d with vitamin A (VAD > VAH) by real-time PCR. Expression of transcripts was normalized to cyclophilin A and ubiquitin C. Relative expression levels in VAC small intestines (A, B) and in VAH small intestines (C) was set at 1.0 for each gene analyzed. Three animals (A, B) and five animals (C) per group were used. *p = 0.02; **p < 0.001.
Discussion

We have demonstrated that during postnatal development RA-producing enzymes are upregulated in MLN-DCs and MLN stromal cells, pointing to environmental factors involved in their 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 signaling, whereas the intake of vitamin A turned out to be instrumental for RALDH activity within the mucosal immune system. RALDH expression in mucosal DCs was critically dependent on vitamin A consumption, and this was also the case for RALDH expression in MLN stromal cells. Notably, RALDH enzyme activity in mucosal DCs could quickly be restored in vitamin A deficient animals by vitamin A supplementation. These data give further insight into 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 intraepithelial 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 wild-type animals in vivo. This suggests that the regulation of RALDH activity in MLN-DCs in vivo does not involve MyD88- and Trif-dependent TLR signaling, confirming the recent findings that MLN-DCs from mice deficient for both MyD88 and Trif still showed Aldefluor activity (47). Furthermore, the literature indicates that DCs that are exposed to intestinal factors express lower TLR levels and are thus less well able to respond to TLR ligands (12, 32, 38, 58, 59). This then further agrees with our finding that MyD88 and Trif are dispensable for RALDH expression in CD103⁺ MLN-DCs in vivo. Nevertheless, we cannot rule out the involvement of other pattern recognition receptors in the induction of RALDH enzyme expression in mucosal DCs.

In contrast, our data showed that vitamin A-derived RA is mandatory for RALDH expression and activity in mucosal 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 the 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 DCs and in MLN-DCs during vitamin A deficiency.

Intestinal epithelial cells produce a vast array of soluble factors that 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, IL-13, TGF-β, thymic stromal lymphopoietin, and IL-4. Of these factors, RA was shown to be crucial for the imprinting of the mucosal DCs phenotype (this article and Ref. 35). At first this seems to be contradicted by statements that RA could only weakly induce RALDH expression (37); however, the concentration used (1 μM RA) was within the range at which we also fail to induce RALDH expression in stromal cells. Both our data and data from others (35) showed that RA used at concentrations ~100 nM can significantly induce RALDH2 expression in BMDCs, 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-BMDCs (37). GM-CSF is produced in the intestines and MLNs by macrophages and granulocytes (37). MLN-DCs from β-c⁻/⁻ animals, lacking the GM-CSFR, showed a reduction of ~50%, whereas the absence of dietary vitamin A resulted in a 94% reduction (Fig. 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 (Supplemental Fig. 4). In addition to GM-CSF, IL-4 and TGF-β were tested in both small intestine and MLNs. Although TGF-β was significantly lower in MLNs from VAD mice, levels of IL-4 and TGF-β were found not to be different in the place where DCs are instructed to express RALDH2 enzymes, that is, the small intestines of VAD mice and VAC mice (Supplemental Fig. 4). We therefore conclude that lack of RALDH2 in lamina propria DCs and consequently in MLN-DCs does not correlate with the absence of GM-CSF, IL-4, and TGF-β in VAD mice.

It is intriguing that RALDH expression in mucosal DCs and stromal cells is induced after birth. Vitamin A-derived RA is crucial for embryonic development, and vitamin A 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 with adult mice (63, 64). Postnatally, newborn mice receive high amounts of vitamin A via breast milk, allowing them to build up vitamin A stores in the liver. Perhaps the first sufficient liver stores need to be generated before vitamin A, bound to its transporter protein RBPs4, can be transported through the blood in sufficient amounts to other tissues. Additionally, the postweaning diet may contain additional dietary factors that further contribute to RALDH expression in mucosal DCs.

During vitamin A deficiency, RALDH expression could still be detected in the small intestines, whereas RARβ expression was virtually absent, indicating that RA-mediated signaling 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, whereas it does not regulate RALDH1 and RALDH2 expression in the small intestine. Other factors, possibly present in the intestinal lumen, may contribute to RALDH expression by epithelial cells. Because 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 important to establish whether RALDH levels in intestinal epithelial cells are constant or whether they are controlled by environmental factors. This may be of particular interest for improving the mucosal immune system in patients in which RALDH function is disturbed, such as Crohn’s disease patients, who have been reported to express low levels of epithelial RALDH1 (36).

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References


