RDH10, RALDH2, and CRABP2 are required components of PPARγ-directed ATRA synthesis and signaling in human dendritic cells

Adrienn Gyöngyösi,* Istvan Szatmari,* Attila Pap,* Balazs Dezső,† Zoltan Pos,‡‡ Lajos Széles,* Tamas Varga,* and Laszlo Nagy¹,*††

Department of Biochemistry and Molecular Biology,* Research Center for Molecular Medicine, University of Debrecen, Medical and Health Science Center, Debrecen, Hungary; Department of Pathology,¹ and MTA-DE “Lendület” Immunogenomics Research Group,‡‡ University of Debrecen, Medical and Health Science Center, Debrecen, Hungary; MTA-SE “Lendület” Experimental and Translational Immunomics Research Group,§ Budapest, Hungary; and Department of Genetics, Cell and Immunobiology,** Semmelweis University, Budapest, Hungary

Abstract All-trans retinoic acid (ATRA) has a key role in dendritic cells (DCs) and affects T cell subtype specification and gut homing. However, the identity of the permissive cell types and the required steps of conversion of vitamin A to biologically active ATRA bringing about retinoic acid receptor-regulated signaling remains elusive. Here we present that only a subset of murine and human DCs express the necessary enzymes, including RDH10, RALDH2, and transporter cellular retinoic acid binding protein (CRABP)2, to produce ATRA and efficient signaling. These permissive cell types include CD103+ DCs, granulocyte-macrophage colony-stimulating factor, and interleukin-4-treated bone marrow-derived murine DCs and human monocyte-derived DCs (mo-DCs). Importantly, in addition to RDH10 and RALDH2, CRABP2 also appears to be regulated by the fatty acid-sensing nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) and colocalize in human gut-associated lymphoid tissue DCs. In our model of human mo-DCs, all three proteins (RDH10, RALDH2, and CRABP2) appeared to be required for ATRA production induced by activation of PPARγ and therefore form a linear pathway. This now functionally validated PPARγ-regulated ATRA producing and signaling axes equips the cells with the capacity to convert precursors to active retinoids in response to receptor-activating fatty acids and is potentially amenable to intervention in diseases involving or affecting mucosal immunity.—Gyöngyösi, A., I. Szatmari, A. Pap, B. Dezső, Z. Pos, L. Széles, T. Varga, and L. Nagy. RDH10, RALDH2, and CRABP2 are required components of PPARγ-directed ATRA synthesis and signaling in human dendritic cells. J. Lipid Res. 2013. 54: 2458–2474.

Supplementary key words retinoid metabolism • retinoic acid receptor signaling • iNKT cells • lipid antigen presentation • lipid transport • cellular retinoic acid binding protein 2 • all-trans retinoic acid • peroxisome proliferator-activated receptor γ

There is an increasing appreciation that metabolic processes contribute to immune cell specification. One of the prime examples of such regulation is the generation and function of all-trans retinoic acid (ATRA) in several cell types of the immune system, primarily in the gut. However, it remains elusive which cell types have the capacity to produce retinoic acid, which genes are required for ATRA biosynthesis and signaling, and which factors contribute to their induction in dendritic cells (DCs).

Abbreviations: APC, antigen-presenting cell; ATRA, all-trans retinoic acid; BM, bone marrow; BM-DC, bone marrow-derived dendritic cell; CRABP, cellular retinoic acid binding protein; Cyp26a1, cytochrome p450 26a1; DEAB, 4-diethyl amino-benzaldehyde; DC, dendritic cell; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; DI, double immunofluorescence; DSS, dextran sodium sulfate; α-GalCer, α-galactosylceramide; FABP4, fatty acid binding protein 4; GALT, gut-associated lymphoid tissue; GM-CSF, granulocyte-macrophage colony-stimulating factor; IF, immunofluorescent; IBD, inflammatory bowel disease; iDC, immature dendritic cell; IHC, immunohistochemistry; IL-4, interleukin-4; iNKT, invariant natural-killer T cell; MDR, medium-chain dehydrogenase/reductase; MLN, mesenteric lymph node; mo-DC, monocyte-derived dendritic cell; Mø, macrophage; NK, natural killer cell; NS, nonsilencing; NS siRNA, nonsilencing control small interfering RNA; ORF, open reading frame; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; PPARγ, peroxisome proliferator-activated receptor γ; RAR, retinoic acid receptor; RARE, retinoic acid response element; RBP, retinol-binding protein; RBP4, RBP4 receptor-2; RSG, rosiglitazone; RT-qPCR, quantitative RT-qPCR; RXR, retinoid X receptor; SDR, short-chain dehydrogenase/reductase; siRNA, small interfering RNA; Sp-DC, splenic dendritic cell; TCR, T cell receptor; TGM2, transglutaminase 2; TLDA, TaqMan low density array.

To whom correspondence should be addressed.
e-mail: nagy@med.unideb.hu

The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of six figures.
DCs have been reported to be important sentinels that play fundamental roles by capturing, processing, and presenting antigens to naive T cells in draining lymph nodes, which elicit antigen-specific immune responses. Under steady-state conditions, the Cx63R1/CD103+ subset continuously migrates to mesenteric lymph nodes (MLNs) and these DCs are involved in maintaining gut tolerance and homeostasis (1, 2). They promote conversion of Foxp3+ regulatory T cells, induce gut-homing receptors CCR9 and α4β7 expression on T and B cells, and provoke T cell-independent IgA switch in naive B cells (3–6). These intestinal immune responses are established by transforming growth factor β along with ATRA, a key cofactor for these processes (7–10). However, the mechanism of ATRA generation in DCs is not fully understood. Retinol, acquired from the diet, can be metabolized to retinol by either the members of the medium-chain dehydrogenase/reductase (MDR) superfamily or by retinol dehydrogenases from the short-chain dehydrogenase/reductase (SDR) superfamily (11). The role of RDH10 for embryonic ATRA synthesis was identified by N-ethyl-N-nitrosourea-induced forward genetic screen. These trex mutant/Rdh10-deficient animals have organ abnormalities and the missense point mutation causes an embryonic lethal phenotype at embryonic day 13.5 (12, 13).

In the second irreversible oxidative step, retinal is further converted to ATRA, catalyzed by RALDH1/ALDH1A1, RALDH2/ALDH1A2, or RALDH3/ALDH1A3 (6, 7, 14). Genetic deletion experiments have proved the physiological contribution of RALDH isoenzymes to ATRA production (15). Raldh2−/− mice show early lethality, suggesting that this enzyme plays indispensable roles in ATRA production in embryos (16–18). Interestingly, these studies also revealed that the sites of expression of RDH10 overlap with those of RALDH2, which suggested that coexpression of both metabolic enzymes were required for ATRA generation. Finally, ATRA binds to the cellular retinoic acid binding proteins (CRABPs), which transport ATRA to the nucleus allowing the activation of retinoic acid receptors (RARs). Upon ligand activation, RARs form heterodimers with retinoid X receptors (RXRs), bind to the retinoic acid response element (RARE), and regulate the transcription of target genes (19–21).

In addition, the activation of another nuclear hormone receptor, peroxisome proliferator-activated receptor γ (PPARγ), can also modulate the immunophenotype of DCs (22). DCs treated with the PPARγ-specific ligand rosiglitazone (RSG) have enhanced lipid antigen presentation capacity (23, 24). DCs have the ability to present lipid antigens such as α-galactosylceramide (α-GalCer) via the cell surface-expressed CD1d to stimulate invariant natural-killer T cell (iNKT) cells that represent a specific T cell population with expressed Natural Killer (NK) cell lineage receptors and semi-invariant CD1d-restricted Va24JaQ T cell receptor-α chain paired with VB11 in humans (25, 26). We have previously shown that PPARγ activation in monocyte-derived DCs (mo-DCs) primes them for ATRA production by the upregulation of RDH10 and RALDH2 expression. The endogenously produced ATRA then induces CD1d cell surface protein expression and triggers intense iNKT expansion (24). However, the requirement of any of these enzymes for ATRA synthesis in DCs has not been proven yet. The work presented here has been based on our previous results and it is a direct extension of it (24). Here we decided to comprehensively characterize the key components that are required for ATRA synthesis and transport in human mo-DCs using genetic as well as pharmacological tools to gain insights into their functional requirements. We have identified CRABP2 as a PPARγ-regulated transcript and characterized the functional contribution of RDH10, RALDH2, and CRABP2 to the enhanced transcriptional activation of ATRA target genes and also to the lipid antigen presentation capacity of mo-DCs. Using a small interfering RNA (siRNA) strategy targeting Rdh10, Raldh2, or CRABP2, we showed that attenuated expression of each of these proteins downregulated the PPARγ-induced CD1d and transglutaminase 2 (TGM2) expression. Furthermore, down-modulated CD1d protein level reduced α-GalCer-activated iNKT expansion in DCT cell coculture experiments.

Furthermore, an immunohistochemistry (IHC) survey of human tissues has revealed that DCs in gut-associated lymphoid tissue (GALT) coexpress PPARγ with RDH10, RALDH2, and CRABP2 in vivo. These data collectively suggest that RDH10 and RALDH2 along with CRABP2 represent a linear pathway, and these genes are functionally required for PPARγ-induced ATRA production and signaling in DCs ex vivo, and likely to play a similar role in vivo as well.

MATERIALS AND METHODS

Ligands

Cells were treated with vehicle control (1:1 of dimethyl sulfoxide/ethanol) or with the following ligands: RSG and GW9662 (Alexis Biochemicals), and ATRA (Sigma), AGN193109 (a gift from Roshantha A. S. Chandraratna, Allergan Inc.), AM580 (Biomol), 4-diethyl amino-benzaldehyde (DEAB) from Fluka, and α-GalCer from Kirin Brewery Ltd. (Gunma, Japan).

Generation of bone marrow-derived DCs

Bone marrow (BM) cells were isolated from the femur of C57BL/6 mice. Animals were housed under specific pathogen free conditions and the experiments were carried out under Committee of Animal Research of the University of Debrecen institutional ethical guidelines and licenses (license number: 21/2011/DEMÁB). BM cells were differentiated to bone marrow-derived DCs (BM-DCs) in RPMI 1640 culturing medium supplemented with 10% FBS (Invitrogen), 500 U/ml penicillin/streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), and 20 ng/ml interleukin-4 (IL-4) or 20 ng/ml GM-CSF alone for 9 days. Cyto- kine treatment was repeated at days 3 and 6. After a 9 day culturing period cells were harvested in Trizol reagent (Invitrogen) for RNA isolation.

Splenic and MLN DC separation

Pooled spleens and MLNs of male C57BL/6 mice were cut into small fragments and digested with collagenase D (Roche) for 40 min at 37°C. Solutions were filtered through a nylon mesh
and washed. Cell suspension was preincubated for 10 min at 4°C with anti-mouse CD16/CD32 mouse BD Fc Block antibody (BD Pharmingen). CD11c+ cells were obtained followed by anti-CD11c MACS bead (Miltenyi Biotec) separation. CD103+ and CD103− DCs were separated by labeling the cells with anti-CD11c-APC and anti-CD103-PE (BD Pharmingen) antibodies and subsequent sorting on FACSVantage (BD Bioscience). Cells were harvested in Trizol reagent (Invitrogen).

DC/Splenocyte coculture experiment

Pooled MLN CD103+ DCs were obtained as described above. We purified splenocytes from pooled spleen of BALB/c mice. Spleens were placed in a Petri dish containing RPMI 1640 medium supplemented with 10% FBS (Invitrogen). Cells were squeezed out with a glass plunger. After washing, we applied Lysing buffer (BD Pharm Lyse, BD Biosciences) against red blood cells. The cell suspension was plated in Petri dishes for 12 h to attach splenic DCs (Sp-DCs). We set the coculture experiment in 12-wells plates, DC/splenoocyte ratio was 1:20, corresponding to 1:10 DC:T cell ratio in 2 ml per well. After 72 h incubation at 37°C, MLN CD103+ DCs were separated by labeling cells with anti-CD11c-APC and anti-CD103-PE (BD Pharmingen) antibodies and subsequent sorting on FACSVantage (BD Bioscience). Cells were harvested in Trizol reagent (Invitrogen).

Human mo-DC culture

Human monocytes (98% CD14+) were isolated from buffy coats of healthy volunteers, obtained with the Regional Ethical Board permit from the Regional Blood Bank, by Ficoll gradient centrifugation, followed by magnetic bead separation using anti-CD14-conjugated microbeads (Miltenyi Biotec). Monocytes were differentiated to DCs at the density of 1.5 × 10⁶ cells/ml in RPMI 1640 medium supplemented with 10% FBS (Invitrogen), 500 U/ml penicillin/streptomycin (Invitrogen), 2 mM l-glutamine (Invitrogen), 500 U/ml GM-CSF (Gentaur Ltd.), and 500 U/ml IL-4 (Peprotech). Cells were cultured for 5 days. Ligands or vehicle control were added to the cell culture at day 0 and at day 3.

RNA interference

siRNA delivery was performed using electroporation of monocytes as described earlier (27). Monocytes were counted and resuspended in Opti-MEM (Invitrogen Life Technologies) with 10% FBS (Invitrogen). Cells were squeezed out with a glass plunger. After washing, we applied Lysing buffer (BD Pharm Lyse, BD Biosciences) against red blood cells. The cell suspension was plated in Petri dishes for 12 h to attach splenic DCs (Sp-DCs). We set the coculture experiment in 12-wells plates, DC/splenoocyte ratio was 1:20, corresponding to 1:10 DC:T cell ratio in 2 ml per well. After 72 h incubation at 37°C, MLN CD103+ DCs were separated by labeling cells with anti-CD11c-APC and anti-CD103-PE (BD Pharmingen) antibodies and subsequent sorting on FACSVantage (BD Bioscience). Cells were harvested in Trizol reagent (Invitrogen).

Aldehyde dehydrogenase activity of mo-DC was assessed using an ALDEFLUOR kit (StemCell Technologies). Cells were incubated at the density of 1 × 10⁵ cells/ml in ALDEFLUOR assay buffer containing activated ALDEFLUOR substrate with or without DEAB for 40 min at 37°C. ALDEFLUOR reactive cells were monitored in FL1 channel of FACSCalibur compared with DEAB-treated control samples.

Expansion of iNKT cells

Mo-DCs were treated with 100 ng/ml of α-GalCer for 48 h to obtain α-GalCer-pulsed DCs. α-GalCer-loaded DCs (1 × 10⁵) were cocultured with monocyte-depleted autologous peripheral blood mononuclear cells (PBMCs) (1 × 10⁶) for 5 days in 24-well plates (1:10 DC/iNKT cell ratio). PBMCs were labeled with anti-TCR Vα24-PE and anti-T cell receptor (TCR) Vβ11-PE monoclonal antibodies (Beckman Coulter), and the double-positive iNKT population was monitored by flow cytometry using FACSCalibur. Additionally, the invariant Vα24-Jα18 (iNKT) TCRα was quantified by using real-time quantitative RT-PCR (RT-qPCR).

RT-qPCR

Total RNA was isolated from cells using Trizol reagent (Invitrogen). One hundred nanograms of total RNA were reverse transcribed with Superscript reverse transcriptase (Invitrogen) and random primers (Invitrogen). This was performed at 42°C for 2 h. Quantitative PCR was performed on LC480 platform (Roche), 40 cycles of 95°C for 10 s and 60°C for 30 s. Gene expression was quantified by the comparative threshold cycle method and normalized to human or mouse Cyclophilin A (PPIA and Ppia) expression as housekeeping gene. All PCR reactions were performed in triplicate. Values are expressed as means ± SD.

Western blot analysis

Twenty micrograms of protein, whole cell lysate, was separated by 12.5% polyacrylamide gel and transferred to PVDF membranes (Millipore). Membranes were probed with anti-CRABP2 (208) antibody, kindly provided by Cecile R. Egly (IGBMC, INSERM, France), and then the membranes were stripped and reprobed with anti-GAPDH antibody (ab78243; Abcam) according to the manufacturer’s recommendations.

Immunoperoxidase staining

For IHC, monocytes, vehicle-treated DCs, or RSG-treated DCs (6 × 10⁵ cells/group) were pelleted and fixed in 4% paraformaldehyde (pH 7.3) for 24 h at 4°C. Cells were then embedded in paraffin, followed by serial sectionings (4 μm). After deparaffinization and dehydration, sections from each cell group were mounted on the same glass slides and were used for peroxidase-based indirect IHC. In brief, sections were treated with 3% H₂O₂ in methanol for 15 min at room temperature to block the endogenous peroxidase. For antigen unmasking, sections were heated in antigen-retrieving citrate buffer (pH 6.0, Dako) for 2 min at 120°C using a pressure cooker. Immunostaining of cells for CRABP2 was carried out using the standard ABC technique utilizing the primary antibody-specific biotinylated secondary antibodies (Vectastain kits, Vector Laboratories). After blocking the nonspecific binding sites, sections were incubated with the primary rabbit antibody to CRABP2 (208) at dilutions of 1 × 1/50 for 1 h at room temperature prior to use of the biotinylated secondary antibodies. The peroxidase-mediated color development was set up for 5 min using the VIP substrate (Vector Labs). Finally, the sections were counterstained with methyl green.
Double immunofluorescence

Double immunofluorescence (DI) was performed on formalin-fixed paraffin-embedded intestinal tissue sections obtained from the archives of surgical specimens of the Department of Pathology, University of Debrecen as described earlier (28). Briefly, following antigen-retrieving and peroxidase block (see previous section), the first primary antibody was visualized with antibody-matched peroxidase-conjugated IgG followed by a tetramethyl-rhodamine-tagged tyramide (Perkin-Elmer) treatment (red fluorescence). After washing and blocking the nonspecific binding sites, sections were incubated with the second primary antibody which was then developed with the use of matched biotinylated secondary antibody (IgG[F(ab)]2) and streptavidin-FITC (Vector Labs, fluorescent isothiocyanate) resulting in green fluorescence. After thorough washings, nuclear counterstaining was made with 4',6-diamidino-2-phenylindole (DAPI) containing the mounting medium (Vector Labs). To check the staining specificities, positive and negative controls were included for each immunofluorescent (IF) reaction as described earlier (28) and as indicated in the Results section.

RESULTS

ATRA biosynthesis in mouse intestinal DCs

As the first step in our studies, we have characterized the expression of the essential enzymes (Fig. 1A) of ATRA biosynthesis and signaling in cells derived from mouse in vivo or ex vivo models. Retinol is transported by retinol-binding protein (RBP), taken up by DCs via retinol binding protein receptor(s) [STRA6 and RBP4 receptor-2 (RBPR2)], and bound to the cellular retinol-binding protein (CRBP) and then serves as substrate for the members of MDR or the SDR superfamily and becomes converted to retinal (Fig. 1A). The second enzymatic step is catalyzed either by RALDH1 or RALDH2. The active metabolite is delivered to the nucleus by CRABP2 and induces the transcription of several retinoid target genes through activating the RAR/RXR heterodimers in mo-DCs. Despite extensive investigation, the components and the exact molecular regulation of this pathway are not completely characterized in either murine or human DCs.

We hypothesized that RDH10 might be the primary enzyme that initiates retinal generation in intestinal DCs and the coexpression of RDH10 and RALDH2 determines the ability of ATRA production in mucosal DCs.

To test this hypothesis, we set out to assess the steps required for ATRA production and signaling in different in vivo- and in vitro-generated DC subtypes (Fig. 1B). First we generated MLN DCs described in the Materials and Methods. The purity of the sorted population was assessed by postsort flow cytometric analysis (supplementary Fig. 1A). We measured the expression level of genes involved in ATRA synthesis and compared the gene expression levels of mRNAs between sorted populations by RT-qPCR. As expected, we could detect Raldh2 only in CD103+ DCs. Rdh10 was expressed in both populations of the MLN DCs, but at an even higher level in the CD103+ cells (Fig. 1C).

Next, we also analyzed other components of the downstream retinoid signaling pathway: we evaluated the gene expression of Rar and Rar isoforms (supplementary Fig. 1A). RAR/RXR heterodimers regulate retinoid target genes such as cytochrome P450 26a1 (Cyp26a1), which had a similar transcription pattern to the Ralldh2 gene (29). Cyp26a1 expression itself is regulated by ATRA through two identified RAREs in the promoter of the gene, suggesting a negative feedback mechanism to control the retinoic acid concentration and active retinoid signaling in cells (30, 31). We further analyzed retinoid signaling by measuring the transcript levels of Tgm2 and Cd1d1, two well-known ATRA target genes (24, 32). CD103+ and CD103− MLN DC populations both expressed Tgm2 and Cd1d1, but the normalized mRNA levels of the genes did not correlate with either Ralldh2 expression or the ATRA production capacity of the cells (Fig. 1C).

In the search for a more suitable mouse DC model for mechanistic studies, we examined ex vivo differentiated cells using TaqMan low density arrays (TLDAs) in additional gene expression analyses. We differentiated GM-CSF-DC or GM-CSF+IL-4-DC from BM (Fig. 1B). To generate a negative control DC population that has no capacity for ATRA production (14), we isolated Sp-DCs from the same mouse strain (C57BL/6). We validated our BM-DC differentiation method by analyzing CD11c DC and F4/80 macrophage marker surface expression and CD11c on Sp-DCs by flow cytometry (supplementary Fig. 1B, C). GM-CSF could elicit Ralldh2 transcription, and the synergistic effect of the two cytokines was confirmed, while Ralldh2 expression in Sp-DCs was barely detectable as it was reported in case of BM-DCs (14). Next we focused on Rdh10 in vivo- and ex vivo-generated cells and found that all DCs expressed this gene (Fig. 1D). A similar expression pattern was observed regarding Cyp26a1.

We compared the gene expression of all Rar (a, b, and g) and Rar (a or b) expressed in mouse DCs (supplementary Fig. 1B). We also analyzed the gene expression of Tgm2 and Cd1d1 in these DC populations. All DC subsets expressed Cd1d1, but it did not show any correlation with retinoid signaling. Tgm2 showed a similar gene expression pattern to Ralldh2, indicating that it could be a reliable marker of active retinoid signaling.

Next we assessed the expression of genes involved in retinol uptake and transport in DCs. However, the only known high-affinity receptor for retinol uptake is STRA6 (stimulated by retinoic acid-6) (33) (34). We could not detect Stra6 gene expression in DC subsets derived from mice (data not shown). The structure of the recently identified RBPR2 is related to human and murine STRA6. Therefore, we hypothesized that RBPR2 could be an alternative receptor in murine and human DCs for retinol uptake. We detected the expression of the Rbpr2 gene in all DC subsets indicating the possibility of retinol uptake to DCs via this alternative receptor (Fig. 1E).

Unexpectedly, the transcription of the Crabp2 gene was not detectable in in vivo subsets (Fig. 1E). The detection of Crabp2 was successful in the case of GM-CSF or GM-CSF+IL-4 DCs in Fig. 1E; therefore, we used the measured Crabp2
**A**

**MLN DCs**

C57BL/6 mice were injected with B16-F10 tumor cell

Bone marrow-derived DCs and splenic DCs

Untreated C57BL/6 mice

**B**

*CD11c*^+^ DCs

*CD103*^+^ DCs

*CD103*^−^ DCs

Bone marrow

GM-CSF-DCs

GM-CSF+IL-4-DCs

Spleen

CD11c*^+^ Sp-DCs

---

**C**

| Gene   | Condition | Norm. mRNA |
|--------|-----------|------------|
| Rdh10  | CD103+    | 0.00012    |
|        | MLN DC    | 0.00009    |
| Raldh2 | CD103+    | 0.00043    |
|        | MLN DC    | 0.00032    |
| Cyp26a1| CD103+    | 0.00001    |
|        | MLN DC    | 0.000002   |
| Cd1d1  | CD103+    | 0.00002    |
|        | MLN DC    | 0.00001    |
| Tgm2   | CD103+    | 0.00003    |
|        | MLN DC    | 0.00002    |

**D**

| Gene   | Condition | Norm. mRNA |
|--------|-----------|------------|
| Rdh10  | GM-CSF-4- | 0.00004    |
|        | IL-4-DC   | 0.00002    |
| Raldh2 | GM-CSF-4- | 0.00006    |
|        | IL-4-DC   | 0.00005    |
| Cyp26a1| GM-CSF-4- | 5e-005     |
|        | IL-4-DC   | 4e-005     |
| Cd1d1  | GM-CSF-4- | 5e-005     |
|        | IL-4-DC   | 4e-005     |
| Tgm2   | GM-CSF-4- | 0.00008    |
|        | IL-4-DC   | 0.00007    |

**E**

| Gene   | Condition | Norm. mRNA |
|--------|-----------|------------|
| Rbpr2  | CD103+    | 0.00012    |
|        | MLN DC    | 0.00009    |
|        | CD103-    | 0.00025    |
|        | MLN DC    | 0.00017    |

**F**

| Gene   | Condition | Norm. mRNA |
|--------|-----------|------------|
| Rbpr2  | CD103+    | 0.00012    |
|        | MLN DC    | 0.00009    |
|        | CD103-    | 0.00025    |
|        | MLN DC    | 0.00017    |

---

**Crabp2**

| Condition | Norm. mRNA |
|-----------|------------|
| GM-CSF-4- | 0.00004    |
| CD103+    | 0.00005    |
| CD103-    | 0.00002    |

---

**Crabp2**

| Condition | Norm. mRNA |
|-----------|------------|
| GM-CSF-4- | 0.00004    |
| CD103+    | 0.00005    |
| CD103-    | 0.00002    |
mRNA level in the GM-CSF+IL-4 DC sample, presented in Fig. 1D, as a technical control of the qPCR assay (labeled with Experiment II/black square in Fig. 1E; the qPCR results of the CD103+ and CD103− MLN-DC samples are labeled with Experiment I/white square). We assessed the role of cellular interactions (T cells) using allogenic splenocytes on Crabp2 and Ript2 gene expression coculturing with ex vivo DCs. In the CD103+/splenocyte coculture experiment, the expression of both Crabp2 and Ript2 was induced in CD103+ DCs, suggesting an enhanced retinol uptake and ATRA delivery as a result of cellular, most likely T cell, interactions (Fig. 1F).

Summarizing these data, three out of five DC types (CD103− MNL DCs, and GM-CSF-DCs or GM-CSF+IL-4-DCs) have a gene expression signature consistent with active ATRA biosynthesis in line with earlier published data (14). Cyp26a1 expression with Ralldh2 appears to indicate active retinoid signaling CD103− MNL DCs, and GM-CSF-DCs or GM-CSF+IL-4-DCs. We found that all DCs express the Rdh10 gene. Based on these gene expression results, we concluded that ATRA biosynthesis is not a universal feature of DCs, and that, in line with our hypothesis, Rdh10 expression overlaps with Ralldh2 expression suggesting that DCs expressing both enzymes are likely to have active ATRA synthesis and signaling.

Characterization of retinoid signaling in human DCs

DCs also exist in the human small intestinal MLNs. Moreover, these DCs have similar functional properties compared to CD103− MLN DCs in mice (36). These data indicated that specific DCs with the ability de novo ATRA synthesis can be present in the human body. Despite much effort and previous work (37), the human DC phenotypes are not identical and easy to match up with the ATRA-producing murine DCs. Therefore we considered using human mo-DCs for mechanistic characterization of the components of retinoid signaling by functional assays. To prove that these ex vivo cells faithfully replicate the behavior of human in vivo DCs with typical DC morphology and characteristic surface markers (23, 38–40), we examined a microarray data set and compared the gene expression pattern of mo-DCs, Langerhans cells, dermal DCs from the skin, CD1c+ DCs from the tonsil, and CD1c− and plasmacytoid DCs (pDCs) from healthy donors (41–44). Our analysis supported the notion that mo-DCs expressed common DC markers as CD83, CD1A, CD14, CD86, CD209, CD36, and ILT7 pDC specific marker such as in vivo subsets (Fig. 2A). We analyzed the cell surface expression of CD14, CD209, and CD11c by FACS measurements. In line with the previously published data, monocytes were found to be CD14+/CD11c−/CD209+ while mo-DCs were CD14−/CD11c+/CD209−, and thus phenotypically resembled in vivo immature dendritic cells (iDCs) (supplementary Fig. IIIA, B).

Next, we examined the gene expression profile of a group of select genes involved specifically in ATRA biosynthesis and signaling (Fig. 2B). RDH10, RDH11, and DHRS9 are expressed in mo-DCs (24). Both RALDH1/ALDH1A1 and RALDH2/ALDH1A2 are expressed at high levels in mo-DCs, while among the in vivo-generated cell types a moderate level of transcription was observed in dermal DCs only, suggesting the possibility of ATRA synthesis in that DC subtype. RALDH3/ALDH1A3 was not expressed in these DC subsets. Among the genes encoding ATRA-transporting proteins, CRABP1 was not expressed, while CRABP2 was expressed ubiquitously. We also analyzed the retinoid signaling by measuring the expression of target genes: TGM2 and CD1D were expressed in tonsillar CD1c+, blood CD1c+, pDC, and in mo-DCs.

Following these analyses we focused on PPARγ and FABP4 expression, the latter is a known marker gene of activated PPARγ signaling (45). We have shown earlier that PPARγ enhances ATRA production by inducing RDH10 and RALDH2 gene expression in mo-DCs differentiated in the presence of the synthetic PPARγ ligand RSG (24). In line with this observation, our microarray data showed that the PPARγ gene is expressed in mo-DCs. The detectable level of FABP4 is likely to indicate either the presence of exogenous PPARγ ligand in the serum or the presence of possible endogenous activators inside cells. Other in vivo-derived DC types failed to express PPARγ or FABP4. This systematic analysis suggested that retinoid signaling is only active in mo-DCs that coexpress RDH10 and RALDH2, and these mo-DCs have PPARγ ligand activation connected to the retinoid signaling pathway.

Next we validated the transcriptional changes of the genes involved in ATRA production during the full differentiation period by RT-qPCR. For this, we differentiated monocytes in the presence of RSG, RSG and GW9662 (a PPARγ antagonist), or GW9662 alone to estimate possible roles of PPARγ in gene expression. We quantified the normalized mRNA level of RALDH genes at an early
timepoint (6 h), and as indicated at later time points (24, 72, and 120 h) (Fig. 2C). In monocytes we detected a high level of RALDH1 that was rapidly downregulated and expressed at elevated level again on the fifth day of differentiation, indicating that RALDH1 may be involved in retinal oxidation in fully differentiated cells. Unlike this gene, the human RALDH2 was barely measurable at the monocyte state. After 6 h, cells expressed RALDH2 at comparable levels to RALDH1. At all later time points, RALDH2 was upregulated in the RSG-treated samples and, except for day 5, ligand treatment induced RALDH2 transcription at similar levels as compared with RALDH1, suggesting that RALDH2 not only has a dominant role in developing DCs but also acts as a metabolizing enzyme in differentiated cells. The RALDH3 isofrom was not detectable irrespective of treatments or time points. As expected, ligand treatment induced the normalized mRNA level of RDH10 after 6 h, indicating that PPARγ activates this gene probably via direct molecular interactions. The expression of RDH10 in RSG-treated samples continuously increased during the entire differentiation period. We detected a similar expression pattern of FABP4. Interestingly, both CRABP2 and TGM2 genes were upregulated in RSG-treated DCs after 24 h in accordance with earlier results (24), CD1d was expressed at a high level in monocytes, but rapidly decreased in cultured cells. Consistent with our previous results, increased CD1d transcription was observed at later time points (23) in RSG-treated samples. PPARγ was immediately induced in differentiating cells, the highest expression was detected at 6 h, and the normalized mRNA level of the gene was detectable at a somewhat lower level in DCs (Fig. 2D).

In summary, ATRA production and signaling is not a universal feature of DCs and it appears to be tightly regulated. We found evidence that ex vivo differentiated mo-DCs express all components required for retinol to ATRA conversion and delivery, suggesting that mo-DCs have the ability for de novo ATRA synthesis and signaling. This ATRA producing capacity can be induced by the coordinate up-regulation of RDH10, RALDH2, and CRABP2.

Transport of ATRA via CRABP2 to the nucleus is PPARγ regulated

As a result of PPARγ activation, we could detect elevated RDH10 and RALDH2 in the cytoplasm of mo-DCs, suggesting that these cells have an enhanced ability to produce retinoic acid (24).

In this part of the study, we aimed to examine whether intracellular ATRA transport could also be regulated by PPARγ. Due to the fact that CRABP2 was also highly induced by PPARγ in RSG-treated mo-DCs, we further examined and characterized its regulation (Fig. 2D). CRABP2 delivers ATRA to the nucleus, thus enhanced expression of CRABP2 should increase the transcriptional activity of RAR (46, 47). Simplistically, CRABP2 would act as a co-activator-like molecule; when it is present, retinoid signaling is more efficient. As shown in Fig. 3A, PPARγ activation profoundly induced the transcript levels of CRABP2. Next we examined if the gene expression changes are manifested at the protein level in mo-DCs. We found that monocytes do not express CRABP2, while control-treated mo-DCs have a detectable level of the protein (Fig. 3B). RSG-treated mo-DCs have a highly enhanced CRABP2 protein level compared with control cells. This appears to be a DC-specific regulation, because monocyte-derived macrophages (MØs) failed to express any CRABP2 (Fig. 3B). We postulated that the elevated CRABP2 expression in PPARγ-instructed DCs might contribute to the enhanced ATRA response. By immunocytochemistry, we further confirmed the elevated CRABP2 expression at the expression site of the delivery protein within mo-DCs. We observed elevated CRABP2 protein expression in DCs as compared with monocytes and a strong upregulation of CRABP2 upon RSG-treatment (Fig. 3C). Based on our immunocytochemistry and Western blot results, we concluded that PPARγ-activated mo-DCs represent a relevant ex vivo model system that appears to be suitable to mechanistically dissect the ATRA biosynthesis and signaling pathway composed of RDH10, RALDH2, and CRABP2 proteins that are coordinately upregulated by PPARγ. We also realized that further investigations are needed to provide direct evidence for CRABP-mediated ATRA transport to the nucleus in these cells.

PPARγ, RDH10, RALDH2, CRABP2, and the ATRA-regulated TGM2 colocalize in DCs of human GALT

In order to provide in vivo relevance to our findings, we systematically surveyed the expression of the components of ATRA biosynthesis and signaling in human tissues. We tested the expression of PPARγ, RDH10, RALDH2, CRABP2, and TGM2 in resting human GALT with no associated intestinal inflammation using in situ IF staining/DI. We chose GALT (for hematoxylin eosin section see Fig. 4B), as this is the most likely place where lipid signaling could contribute to DC differentiation and subtype specification in the gut. As shown in Fig. 4A (representing an IF image), we observed that PPARγ could be readily detected in white adipose tissue (WAT; positive control to ensure the specificity of the antibody during IF staining). DI of resting GALT for PPARγ (red) demonstrates that PPARγ is in part...
coexpressed with Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (CD-SIGN) (green cytoplasmic, arrows) in mucosal lymphoid tissue cells that have cytoplasmic projections in a network pattern indicating DC phenotype (Fig. 4C). Interestingly, nuclear PPARγ (red) and the cytoplasmic TGM2 proteins (green) show co-expression in similar cells of GALT exhibiting cytoplasmic green projection characteristic of DC elements, comparable with the staining pattern as seen for PPARγ-DC-SIGN of image C. Therefore, these cells coexpressing PPARγ/TGM2 should represent the DC population of GALT, similarly to PPARγ/DC-SIGN positive cells (Fig. 4D). These data indicate that in resting lymphoid tissues some of the PPARγ-positive DCs express TGM2 simultaneously, suggesting that PPARγ might regulate ATRA-dependent transcription in vivo as well. On the other hand, in GALT we showed that some PPARγ (red) positive cells coexpressed RDH10 (green cytoplasm) (Fig. 4E). Similarly, we observed few PPARγ-expressing DCs with RALDH2 and CRABP2 coexpression, respectively (Fig. 4F, G). RDH10, RALDH2, and CRABP2 (green cytoplasmic and/or nuclear) also colocalized with DC-SIGN (red cytoplasmic projections) in some mucosal DCs (Fig. 4H–J). Note that the number of RDH10-, RALDH2-, and CRABP2-positive cells are roughly the same when Fig. 4E compared to Fig. 4H–J indicating non-activated DC state in resting GALT. However, the number of PPARγ positive cells are increased in cases of inflammatory bowel diseases (IBDs) (data not shown).

These data collectively strongly suggest that the key components of ATRA synthesis and the PPARγ are expressed together in some of the antigen-presenting cells (APCs) of the mucosal lymphoid tissues, consistent with a previous report which demonstrated that murine intestinal DCs expressed RALDH2 (6).

Increased RALDH activity in PPARγ-activated mo-DCs

We wanted to provide functional evidence that indeed retinoic acid biosynthesis takes place in mo-DCs. Using a sensitive and quantitative liquid chromatography-mass spectrometry (LC-MS) method, we previously reported that mo-DCs have the ability to produce ATRA by oxidation of retinal in a PPARγ-dependent manner (24). We aimed to further investigate this result and the function of cellular RALDHs using the ALDEFLUOR staining assay that is suitable to detect enzymatic activity of RALDHs inside the cells. Cells were differentiated (as described in the Materials and Methods) in the presence of DMSO/ethanol (C-Control) RSG, or RSG+GW9662. At 120 h, the cells were divided and incubated with fluorescent ALDEFLUOR, a substrate for RALDHs, either in the absence or the presence of DEAB, a specific RALDH inhibitor. After 40 min incubation, RALDH activity was measured by flow cytometry. There were 8% RALDH positive cells among vehicle-treated DCs (Fig. 5). In the presence of RSG, the number of RALDH positive cells was increased to 40%. We noted that a much larger enzyme activity was displayed in these treated DCs than even in the positive ones in control cells. In the RSG and GW9662 cotreated sample, the RALDH activity was similar to vehicle-treated control DCs.

Next, we assessed RALDH activity in mo-DCs electroporated at monocyte stage using specific siRNAs against siRDH10, siRALDH2, and siCRABP2 and NS-scrambled control siRNA. We treated cells with DMSO/ethanol (C), RSG, and RSG+GW9662. At day five ALDEFLUOR activity was quantified. We measured lower RALDH activity only in the siRALDH2 electroporated sample (supplementary Fig. IV).

These results suggest that RALDHs are active in mo-DCs, and the enhanced ATRA production capacity of mo-DCs is PPARγ dependent. Interestingly, we could detect heterogeneity in this respect in the RSG-mo-DC population, but the generated endogenous ATRA level is at the range of RAR activation.

PPARγ activation induces RAR signaling/gene expression via RDH10, RALDH2, and CRABP2

Based on these data, one can hypothesize that RDH10, RALDH2, and CRABP2 might be required for PPARγ-regulated ATRA synthesis and gene expression. Despite the murine DC results (37), the model one can test this in is the human mo-DC. PPARγ activation leads to transcriptional activation of several RAR target genes in human mo-DCs (24). Pharmacological analysis revealed that administration of the RALDH inhibitor DEAB reduced gene expression of CD1D and TGM2 upon RSG treatment, suggesting the importance of RALDH2 in PPARγ-enhanced retinoid signaling (24). We have extended our studies by testing to determine whether the oxidizing enzymes and CRABP2 are indeed mechanistically indispensable for retinoid-regulated gene expression induced by PPARγ. For this, we decided to use a siRNA-based approach. We delivered siRNA against RDH10, RALDH2, or CRABP2 and FABP4 (as a control) to CD14+ monocytes after cell separation via electroporation, and differentiated cells as described in the Materials and Methods. RSG was administered as indicated in Fig. 6. After 24 or 48 h of RSG treatment, we assessed the transcript level of CD1D and TGM2 using RT-qPCR. We observed that PPARγ-induced CD1D expression was down-modulated by all except FABP4-specific siRNA at both indicated time points (24 and 48 h). TGM2 expression changes were similar at 24 h, but only RDH10-specific siRNA inhibited it significantly at 48 h as compared with nonsilencing control treated (NS) DCs (Fig. 6A). We quantified the gene expression of RALDH2 by RT-qPCR. Only siRALDH2 could significantly reduce the normalized mRNA level of RALDH2 as expected (supplementary Fig. V).

These data suggest that RDH10 is a key component of retinol conversion, and PPARγ-mediated retinal oxidation is catalyzed by RALDH2.

In the next set of experiments, we electroporated monocytes with RDH10-specific siRNA and treated as described in Fig. 6B; then we measured CD1d cell surface protein expression by flow cytometry. Transient RDH10 siRNA transfection reduced CD1d levels on DCs and it was still down-regulated at day 5 post-electroporation (Fig. 6B). These results strongly suggested that PPARγ-mediated signaling induced retinol conversion by induced RDH10 in mo-DCs. The produced retinal is oxidized to ATRA by RALDH2.
Retinoid signaling in DCs

During iNKT expansion, the mRNA expression of the invariant Vα24-Jβ11 (iNKT) TCR marker gene correlates with the cell surface expression of TCR Vα24 and TCR Vβ11 (48). We validated the RT-qPCR measurements on iNKT cells expanded by α-GalCer-loaded control or RSG-treated mo-DCs (Fig. 7B). Therefore, we studied the functional consequence of RDH10, RALDH2, and CRABP2 silencing on iNKT expansion by measuring Vα24-Jβ11 (iNKT) TCR gene expression as described in Fig. 6B. α-GalCer-pulsed mo-DCs have displayed enhanced Vα24-Jβ11 (iNKT) TCR gene expression further inducible by RSG administration as compared with control (NS) treated cells. Next, siRNA-treated mo-DCs were loaded with α-GalCer lipid antigen and cocultured for 5 days. As shown in Fig. 7C, siRNA against RDH10, RALDH2, and CRABP2 enzymes reduced the normalized TCR VA24 mRNA levels in RSG-treated samples as compared with nonsilencing control (NS) treated cells. Our data revealed significantly reduced Vα24-Jβ11 (iNKT) TCRα transcription (Fig. 7C).

Based on these functional results, we could conclude that PPARγ is acutely involved in retinoid signaling via inducing endogenous ATRA production. The primary enzyme for retinol oxidation is RDH10. The RSG-treated cells have the ability to synthesize ATRA because the coexpression of RDH10 and RALDH2 in the cells allows it. Moreover, retinoid signaling is more effective in the presence of PPARγ-induced CRABP2 that transports ATRA to the nucleus.

The enhanced retinoid signaling was more effective in the presence of the CRABP2 ATRA transporter. In the nucleus, ATRA activates regulated target genes via RAR/RXR heterodimers due to integrated PPARγ-RAR signaling.

PPARγ-induced iNKT expansion is attenuated by RDH10, RALDH2, or CRABP2 knockdown

Next we aimed to assess the functional consequence of RDH10, RALDH2, and CRAB2 in in vitro functional assay. The lipid antigen-presenting capacity of PPARγ-activated DCs is induced and mediated through the upregulated cell surface protein expression of CD1d protein (23, 24, 48). Human iNKT cells respond to α-GalCer, a lipid antigen restricted exclusively to CD1d molecules. First, we sought to investigate whether RDH10 can influence the PPARγ-mediated iNKT expansion capacity of the APCs. For this, we silenced the RDH10 gene in monocytes with RDH10-specific siRNA or we used a nonsilencing control siRNA (NS siRNA) as described above for Fig. 5A. Cells were differentiatied to mo-DCs in the presence of DMSO/ethanol for the control-treated sample or RSG for PPARγ activation and cells were pulsed with or without α-GalCer for 48 h and then cocultured with autologous PBMCs at a 1:10 ratio. The iNKT proliferation capacity was monitored by Vα24/Vβ11 double staining. As expected, enhanced iNKT expansion was detected in RSG-treated and NS siRNA-transfected samples, while reduced iNKT cell numbers were observed in RDH10 siRNA-treated cells (Fig. 7A).
Fig. 4. DI staining analysis of ATRA biosynthesis and the putative signaling pathways along with PPARγ expression in human GALT. A: Positive control for PPARγ IF staining: the nuclei of white adipose tissue (WAT) cells show characteristic expression [lighting-red fluorescence (arrows) is magnified in the insert]. B: A representative area of hematoxylin eosin-stained human GALT is shown where arrows point to the mucosa-associated lymphoid tissue (M, mucosa; L, lumen). C: Using double IF, the same tissue shows that the PPARγ protein (nuclear red fluorescence) is in part coexpressed with DC-SIGN (green cytoplasmic, arrows) in mucosal lymphoid tissue cells that have exhibiting cytoplasmic projection in a network pattern indicating DC phenotype. D: The nuclear PPARγ (red) and the cytoplasmic TGM2 protein (green) show coexpression in the similar cells of GALT exhibiting cytoplasmic green fluorescence characteristic of DC elements comparable with the staining pattern as seen for the PPARγ-DG/SIGN of image (C). E: Some of the DCs exhibiting PPARγ (red nuclei) also express RDH10 (green cytoplasm) in resting GALT. F: Rarely, PPARγ (red nuclei) is also colabeled with RALDH2 (green cytoplasm) in the same GALT. G: Also scattered cells with PPARγ positivity (red nuclei) show simultaneous expression with CRABP2 (green cytoplasm, arrows) in the same GALT. H: In many DC-SIGN-positive DCs (red cytoplasm) there is a RDH10 (green nucleic and cytoplasmic) coexpression (arrows). I: DC-SIGN expressing DCs (cells with red cytoplasmic projections) may show coexpression with RALDH2 (arrow) as well (cells with green nuclei or cytoplasm). J: Few DC-SIGN-positive cells (red cytoplasm) show colocalization with CRABP2 protein (green nuclei and cytoplasm), also (arrow) indicating that CRABP2-positive cells are of DC type within resting GALT. [Except for image (B), all are DI photographs with DAPI nuclear counterstaining. Original magnifications: (A, C, D), 20×; (B), 10×; (E–G), 40×; (H–J), 63×.]
DISCUSSION

It is increasingly accepted that several links connect immune functions of the whole body to cellular metabolism. One of the most striking examples of such a regulatory role is the involvement of vitamin A/retinoid metabolism and ATRA production in the context of cell type specification and immune regulation. ATRA’s role is primarily linked to the gut and mucosal immunity.

Intestinal homeostasis is critically controlled by the interaction between immune cells, epithelial cells, and strains of commensal bacteria. The balance between immune reactivity and tolerance under steady-state and pathogenic conditions is primarily regulated by the immunoregulatory properties of GALT-associated DCs. The main goal of our study was to systematically survey mouse and human DCs for ATRA production and signaling, and to identify and validate additional key regulatory components of endogenous ATRA synthesis in human and mouse DCs.

The endogenous ATRA generation capacity in intestinal DCs was related to the expression of intracellular RALDHs. Although previous works have demonstrated that murine DCs express several Raldh isoforms (7, 10, 14, 24, 49–52), the functional contribution of these isoforms to ATRA synthesis remain to be determined and the prior enzymatic step has not been evaluated in these cell types at all.

For ATRA generation, retinol is believed to be converted to retinol by members of the aldehyde dehydrogenases. Alternatively, enzymes of the SDR superfamily may also participate in this process (6). Genetic evidence that RDH10 is indispensable for embryonic retinol metabolism has been provided (12, 13) and pinpointed to this enzyme as the key mediator of this conversion. Furthermore, intracellular localization of RDH10 and RALDH2 could be correlated (16–18). These results suggested that RDH10 might be the key enzyme that could initiate retinol metabolism in intestinal DCs, and coexpression of RDH10 and RALDH2 is essentially required for the ability of mucosal CD103+ DCs to generate de novo ATRA. We tested this hypothesis first by using MLN DCs isolated from C57BL/6 mice, Sp-DCs from untreated mice, and ex vivo-differentiated BM-DCs in the presence of GM-CSF or GM-CSF and IL-4 (14). Using RT-qPCR and TLDA methods, we assessed that the transcription of the Rdh10 gene was detectable in all examined DC subtypes and that the expression of Rdh10 coincides/overlaps with Ralhd2. We could show clear correlation between the ATRA-generating capacity of in vivo- and in vitro-produced DCs and the expression of the Cyp26a1 gene as a reliable marker for active retinoid signaling in intestinal DCs.

We also assumed that ATRA would influence the transcription of some of the known target genes identified in human DCs. Interestingly, we did not observe correlations between Tgm2 and Cd1d genes and ATRA-producing capacity in in vivo subsets, particularly in the most widely characterized CD103+ cells.

Moreover, the gene encoding ATRA transporter Crabp2 was also barely or not detectable in DCs of different origin. An important question is how ATRA is transported in DCs. These transport mechanisms in MLN DCs are not characterized. ATRA can be delivered to the nucleus by CRABP2 that activates retinoid signaling via RAR/RXR heterodimers or by FABP5 molecules that enhance the ligand-induced transcriptional activities of PPARβ/δ heterodimers (47, 53). In MLN CD103+ DCs Crabp2 is not expressed, but under the culture condition the expression of Crabp2 mRNA was induced in sorted MLN DC103+ DCs suggesting that T cells most likely activate Crabp2 transcription in DCs. The signaling details of this induction require further investigations. We assessed the expression of Crabp1 in in vivo and in vitro DC subsets as well. According to our RT-qPCR measurement, Crabp1 is not expressed in these DCs. Based on these results, one might conclude that there might be other ATRA-delivering proteins in some of the mouse DCs.

The difference between human and murine DCs in this respect is apparent. Nuclear receptor-mediated signals might be involved in RALDH expression in intestinal DCs, leading to a specific subtype that not only stores and carries, but also produces a significant level of ATRA. However, agonists of PPARγ did not significantly induce Rdh10 expression in Flt3L-generated BM-DCs and purified Sp-DCs compared with the effect of IL-4 or IL-13 (14). These apparent discrepancies between the mouse and human DCs remain to be clarified. We remain of the opinion that there is not sufficient evidence to suggest that PPARγ is able to regulate retinoid biosynthesis in murine DCs. We proved earlier that human monocytes expressed PPARγ receptors and that the receptors are active under ex vivo culture conditions (23). Systematic microarray analyses also revealed that the activation of PPARγ by RSG regulated the expression of genes contributing primarily to fatty acid uptake, transport, lipid storage, and attenuated lipid metabolism (43). Moreover, the PPARγ-regulated lipid metabolic pathways could be associated with the altered immune response of DCs (23, 24, 48). PPARγ enhanced the lipid antigen presentation capacity of DCs via CD1d.
Fig. 6. Expression of RDH10, RALDH2, and CRABP2 is required for PPARγ-induced retinoid signaling and gene expression in mo-DCs. A: Monocytes were electroporated with siRNA against *RDH10*, *RALDH2*, *CRABP2*, and *FABP4*. NEP, no electroporated control sample; NS, nonsilencing control sample electroporated with scrambled control siRNA. Gene expression of *CD1D* was determined by RT-qPCR. Means normalized to Cyclophilin A ± SD; n = 3; **NS-RDH10 24 h (P = 0.0062); **NS-RALDH2 24 h (P = 0.0074); *NS-CRABP2 24 h (P = 0.0161), NS-FABP4 24 h (P = 0.4082); ***NS-RDH10 48 h (P = 0.0005); **NS-RALDH2 24 h (P = 0.00713); **NS-CRABP2 24 h (P = 0.0014), NS-FABP4 24 h (P = 0.2188). Gene expression of *TGM2* was determined by RT-qPCR. Means normalized to Cyclophilin A ± SD; n = 3; *NS-RDH10 24 h (P = 0.0119); *NS-RALDH2 24 h (P = 0.0111); *NS-CRABP2 24 h (P = 0.0346), NS-FABP4 24 h (P = 0.2539); **NS-RDH10 48 h (P = 0.0030), NS-RALDH2 24 h (P = 0.2974), NS-CRABP2 24 h (P = 0.3895), NS-FABP4 24 h (P = 0.0854). B: Expression of CD1d protein on 5-day mo-DCs was measured by FACS analysis. Monocytes were electroporated with scramble control siRNA (NS) and siRNA against *RDH10* (siRDH10). mo-DCs were differentiated in the presence of DMSO/ethanol (C=Control) or 2.5 μM RSG (RSG).
Fig. 7. RDH10 is required for PPARγ-induced iNKT activation in mo-DCs. A: Expansion of iNKT cells was assessed in lipid antigen-presentation assay. Monocytes were obtained from buffy coats of healthy donors. Monocytes were electroporated with siRNA against RDH10 and scramble control siRNA (NS). Cells were differentiated in the presence of DMSO/ethanol (C=Control) or 2.5 μM RSG (RSG) for 5 days. iNKT differentiation was detected by TCR Vα24/TCR Vβ11 double staining utilizing FACS analysis. B: iNKT expansion was quantified by RT-qPCR. Means normalized to Cyclophilin A ± SD. C: Expression of Vα24-Jα18 (iNKT) TCRα gene was quantified by RT-qPCR. Monocytes were electroporated with siRNA against RDH10, RALDH2, and CRABP2. NS, nonsilencing control sample electroporated with scrambled control siRNA. mo-DCs were differentiated in the presence of DMSO/ethanol (C=Control) or 2.5 μM RSG (RSG) for 5 days. Means normalized to Cyclophilin A ± SD; n = 3; **NS-RDH10 (P = 0.0016); *NS-RALDH2 (P = 0.0315); ***NS-CRABP2 (P = 0.0003).
molecules (23, 24, 48), which is essential for ligand presentation and recognition by the iNKTs (26, 54, 55).

In this cellular context, we have found that activation of PPARγ initiates a transcriptional program of altered lipid metabolism in mo-DCs via induced expression of genes that are required for endogenous ATRA production (24). Importantly, we assumed that retinol is metabolized to retinal primarily by RDH10 in this process. The mRNA level of RDH10 was increased by PPARγ after 6 h, suggesting that this gene, like FABP4, could be regulated directly by PPARγ, while the direct regulatory function and binding of PPARγ/RXR via a response element in the RDH10 promoter still needs to be investigated. mo-DCs express the key enzymes (RALDHs) of ATRA production. ATRA activated RARα receptors and induced CD1d expression at mRNA and protein levels. These results suggested that transcriptional events in human mo-DCs that upregulate the CD1d gene are coordinately mediated by PPARγ and RARα receptors (23, 24).

We characterized the expression of genes responsible for retinol uptake into murine and human DCs. We could not detect STRA6 expression in mo-DCs, the only known high-affinity receptor for RBP4 (33, 34), as we expected based on our microarray results (data not shown). We have similar results in the case of murine DCs. The structure of RBPR2, a novel retinol transporter, is related and highly conserved between human and murine STRA6 (35). Therefore, we hypothesized that RBPR2 could be an alternative receptor in murine and human DCs for retinol uptake. We detected Rbp2 gene expression in a different subset of murine DCs. This gene was upregulated in MLN CD103+ DCs under coculture conditions. The human ortholog of RBPR2 is localized at two separate sites (RBPR2A and RBPR2B) of chromosome 9’s p and q arms (35). The homology between murine Rbp2 and the two human ortholog Open Reading Frames (ORFs) suggests the possibility that if these ORFs are transcribed, they can form functional protein on the DC surface. The expressions of human RBPR2A and RBPR2B were assessed and presented in supplementary Fig. VI. The expression of RBPR2A could be detected; however, this was highly donor-dependent and not altered by PPARγ. RBPR2B was also expressed by DCs, but the normalized mRNA level of this fragment was much lower compared to RBPR2A. Additional RBP-binding assays would be required to confirm whether RBPR2A and RBPR2B can form a functional receptor on DCs.

Based on these and our previous results we chose mo-DCs as our model system to test the functional contribution of the above enzymes for ATRA synthesis and signaling. This approach proved to be successful and validated the functional requirement suggested by the gene expression studies not only at the level of gene expression, but also in T cell activating function.

An additional finding of this work is that not only ATRA synthesis can be regulated by PPARγ but also the delivery of the active metabolite to the nucleus. We found that CRABP2 transporter protein is upregulated upon RSG-treatment, suggesting that DCs with activated PPARγ have enhanced ATRA transport to the nucleus. We showed earlier that RDH10 and RALDH2 proteins were expressed in mo-DCs and were upregulated upon RSG-treatment (24), and by using CRABP2-detecting IHC analysis, we confirmed that mo-DCs are a relevant ex vivo model for investigating DC ATRA producing capacity and transport. Direct evidence for CRABP-mediated ATRA transport to the nucleus requires further experimentation.

To date our knowledge is still limited about the nature of in vivo ATRA-producing APCs in the human intestinal system. GALT is the most likely place where intensive lipid absorption occurs and PPARγ activators can be generated. Therefore it is reasonable to assume that lipid signaling could participate in DC subtype specification in these lymphoid tissues. Our IHC/DI results clearly suggested that DC-SIGN positive, GALT-associated immune cells readily express the key components of ATRA producing and signaling. In addition, PPARγ-positive DCs coexpressed TGM2, strongly suggesting that these cells have an active retinoid signaling system and represent an in vivo relevant ATRA-producing cell type. Presumably our ex vivo mo-DCs may correspond to these in vivo DC/macroage-like APCs.

The question of whether PPARγ-induced ATRA generated by mucosal DCs is physiologically relevant remains to be answered. However, a link to the prevention of human IBD can be established. PPARγ was shown to be associated with IBD in a mouse model of experimental colitis (56–58). Genetic evidence of PPARγ-mediated protection against colon inflammation was shown in PPARγ heterozygous mice (Pparg+/−), and targeted disruption of the Pparg gene in intestinal epithelial cells enhanced the susceptibility to dextran sodium sulfate (DSS)-induced colitis (59). RSG treatment and other PPARγ ligands attenuate the severity of colitis in both intestinal epithelial cell and macrophage-specific PPARγ mutant mouse strains (57, 60). Due to potential side effects, the application of RSG in the treatment of IBD is not likely (61, 62). Efforts toward the discovery of a new class of PPARγ agonist that elicits therapeutic effects against IBD with limited or no adverse side effects revealed that conjugated linoleic acid (CLA) is a safer alternative to RSG in the model of spontaneous pan-enteritis and DSS-induced colitis (63–65).

Future studies should assess the role of DC-expressed PPARγ in IBD and potentially in other diseases. Our experimental results can be useful to design an even more effective treatment in the prevention or amelioration of ulcerative colitis and Crohn’s disease.

In summary, we have established a linear relationship between RDH10, RALDH2, and CRABP2 in PPARγ-induced ATRA synthesis and signaling that showed the requirement of these proteins. Further studies are needed to dissect the regulation of these pathways and ways to modulate their expression and activity. These new pathways could provide insight into how ATRA production could shape the immune response, and could potentially be used to treat diseases affecting mucosal immunity.

The authors would like to acknowledge the excellent technical help of Ms. Ibolya Furtos and Marta Beladi. The authors are
indebted to Dr. Á. Lányi and the members of the Nagy laboratory for discussions and comments on the manuscript.

REFERENCES

1. Johansson-Lindbom, B., M. Svensson, O. Pabst, C. Palmquist, G. Marquez, R. Forster, and W. W. Agace. 2005. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. J. Exp. Med. 202: 1063–1073.

2. Schulz, O., E. Jaensson, E. K. Persson, X. Liu, T. Worbs, W. W. Agace, and O. Pabst. 2009. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. J. Exp. Med. 206: 3101–3114.

3. Hammerschmidt, S. I., M. Friedrichsen, J. Boelter, M. Lyszkievicz, E. Kremmer, O. Pabst, and R. Forster. 2011. Retinoic acid induces homing of protective T and B cells to the gut after subcutaneous immunization in mice. J. Clin. Invest. 121: 3051–3061.

4. Mora, J. R., M. Iswata, Y. Eshima, H. Kagechika, C. Kato, and S. Y. Song. 2004. Retinoic acid imprinting gut-homing specificity on T cells. Immunity. 21: 527–538.

5. Coombes, J. L., K. R. Siddiqui, C. V. Arancibia-Carcamo, J. Hall, C. M. Sun, Y. Belkaid, and F. Powrie. 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J. Exp. Med. 204: 1757–1764.

6. Sato, A., M. Hashiguchi, E. Toda, A. Iwasaki, S. Hachimura, and S. Kaminogawa. 2003. CD11b+ Peyer’s patch dendritic cells secrete IL-6 and induce IgA secretion from naive B cells. J. Immunol. 171: 3684–3690.

7. Sun, C. M., J. A. Hall, R. B. Blank, N. Bouladoux, M. Oukka, J. R. Kumar, L. L. Sandell, P. A. Trainor, F. Koentgen, and G. Duester. 2012. Alcohol and aldehyde dehydrogenases: retinoid metabolic effects in mouse knockout models. J. Leukoc. Biol. 91: 1250–1265.

8. Kramer, K., V. Subbarayan, P. Dolle, and P. Chambon. 1999. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. Nat. Genet. 21: 444–448.

9. Niederreither, K., J. Vermot, N. Messaddeq, B. Schuhbaur, P. Chambon, and P. Dolle. 2001. Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. Development. 128: 1019–1031.

10. Chambon, P. 1996. A decade of molecular biology of retinoic acid receptors. FASEB J. 10: 940–954.

11. Mangelsdorf, D. J., and R. M. Evans. 1995. The RXR heterodimers and orphan receptors. Cell. 83: 841–850.

12. Umesono, K., V. Giguere, C. K. Glass, M. G. Rosenfeld, and R. M. Evans. 1988. Retinoic acid and thyroid hormone induce gene expression through a common responsive element. Nature. 336: 262–265.

13. Mangelsdorf, D. J., C. Thummler, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, et al. 1995. The nuclear receptor superfamily: the second decade. Cell. 83: 835–839.

14. Szyman, I. P., Gogolak, J. S. Im, B. Dezo, E. Rajnagyolvi, and L. Nagy. 2004. Activation of PPARgamma specifies a dendritic cell subtype capable of enhanced induction of INKT cell expansion. Immunity. 21: 95–106.

15. Schatz, I. A. Pap, R. Ruhl, J. X. Ma, P. A. Blizard, G. S. Besra, E. Rajnagyolvi, B. Dezo, and L. Nagy. 2006. PPARgamma controls CD1d expression by turning on retinoic acid synthesis in developing human dendritic cells. J. Exp. Med. 205: 2531–2562.

16. Bendelac, A., P. B. Savage, and L. Teyton. 2007. The biology of NKT cells. Annu. Rev. Immunol. 25: 297–336.

17. Porcelli, S., and R. L. Modlin. 1999. The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. Annu. Rev. Immunol. 17: 297–329.

18. Schaaf, N., J. Dorrie, P. Thumann, V. E. Beck, I. Muller, E. S. Schultz, E. Kampgen, D. Dieckmann, and G. Schuler. 2005. Generation of an optimized polyvalent monocoyte-derived dendritic cell vaccine by transfecting defined RNAs after rather than before maturation. J. Immunol. 174: 3087–3097.

19. Gogolak, P., B. Rethi, I. Szatmari, A. Lanyi, B. Dezo, L. Nagy, and E. Rajnagyolvi. 2007. Differentiation of CD1d+ and CD1d- monocyte-derived dendritic cells is biased by lipid environment and PPARgamma. Blood. 109: 643–652.

20. White, J. A. Y. Guo, K. Baetz, B. Beckett-Jones, J. Bonasoro, K. E. Hsu, F. J. Dilworth, G. Jones, and M. Petkov.1996. Identification of the retinoic acid-inducible all-trans-retinoic acid 4-hydroxylase. J. Biol. Chem. 271: 29922–29927.

21. Loudig, O. G. A. Maclean, N. L. Dore, L. Lau, and M. Petkov. 2005. Transcriptional co-operativity between distant retinoic acid response elements in regulation of Cyp26A1 inducibility. Biochem. J. 392: 241–248.

22. Ray, W. J. G. Bain, M. Yao, and D. I. Gottlieb. 1997. CYP26, a novel mammalian cytochrome P450, is induced by retinoic acid and defines a new family. J. Biol. Chem. 272: 18702–18708.

23. Chiocca, E. A., P. J. Davies, and J. P. Stein. 1988. The molecular basis of retinoic acid action. Transcriptional regulation of tissue transglutaminase gene expression in macrophages. J. Biol. Chem. 263: 11584–11589.

24. Kawaguchi, R., J. Yu, J. Honda, J. Hu, J. Whitelegg, P. Ping, P. Wiita, D. Bok, and H. Sun. 2007. A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. Science. 315: 820–825.

25. Sun, H., and R. Kawaguchi. 2011. The membrane receptor for plasma retinol-binding protein, a new type of cell-surface receptor. Int. Rev. Cell Mol. Biol. 288: 1–41.

26. Alapatt, P., F. Guo, S. M. Komanetsky, S. Wang, J. Cai, A. Sargyan, E. Rodriguez Diaz, B. T. Bacon, P. Ayal, and T. E. Graham. 2013. Liver retinol transporter and receptor for serum retinol-binding protein (RP4). J. Biol. Chem. 288: 1250–1265.

27. Jaensson, E., H. Uronen-Hansson, O. Pabst, B. Eksteen, J. Tian, J. L. Coombes, P. L. Berg, T. Davidsson, F. Powrie, B. Johansson-Lindbom, et al. 2008. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. J. Exp. Med. 205: 2139–2149.

28. Housley, W. J., C. A. O’Conor, F. Nichols, L. Puddington, E. G. Lingenheld, L. Zhu, and R. B. Clark. 2009. PPARgamma regulates retinoic acid-mediated DC induction of Tregs. J. Leukoc. Biol. 86: 293–301.

29. Gosset, P., A. S. Charbonnier, P. Delerive, J. Fontaine, B. Baels, J. P. J. Van, A. B. Tonnel, and V. Trottein. 2001. Peroxisome proliferator-activated receptor gamma activators affect the maturation of human monocyte-derived dendritic cells. Eur. J. Immunol. 31: 2857–2865.

30. Gallusso, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin
4 and downregulated by tumor necrosis factor alpha. J. Exp. Med. 179: 1109–1118.
40. Thurner, B., C. Roder, D. Dieckmann, M. Heuer, M. Kruse, A. Glaser, P. Keikavousi, E. Kampgen, A. Bender, and G. Schuler. 1999. Generation of large numbers of fully mature and stable dendritic cells from leukapheresis products for clinical application. J. Immunol. Methods. 235: 1–15.
41. Lindstedt, M., K. Lundberg, and C. A. Borrebaeck. 2005. Gene family clustering identifies functionally associated subsets of human in vivo blood and tonsillar dendritic cells. J. Immunol. 175: 4839–4846.

Santegoets, S. J., S. Gibbs, K. Kroeze, R. van de Ven, R. J. Scheper, C. A. Borrebaeck, T. D. de Grujil, and M. Lindstedt. 2008. Transcriptional profiling of human skin-resident Langerhans cells and CD1d+ dermal dendritic cells: differential activation states suggest distinct functions. J. Leukoc. Biol. 84: 143–151.
43. Szatmari, I., D. Toroescu, M. Agostini, T. Nagy, M. Gurnell, E. Barta, K. Chatterjee, and L. Nagy. 2007. PPARgamma regulates the function of human dendritic cells primarily by altering lipid metabolism. Blood. 110: 3271–3280.
44. Széles, L., S. Pólisha, G. Nagy, I. Szatmari, A. Szanto, A. Pap, M. Lindstedt, S. J. Santegoets, R. Rühl, B. Dezso, et al. 2010. Research resource: transcriptome profiling of genes regulated by RXR and its permissive and nonpermissive partners in differentiating monoocyte-derived dendritic cells. Mol. Endocrinol. 24: 2218–2231.
45. Tontonoz, P., E. Hu, R. A. Graves, A. I. Budavari, and B. M. Tontonoz, P. , E. Hu , R. A. Graves , A. I. Budavari , and B. M. Tontonoz . 1999 . Distinct
46. Illarionov, B. Dezso, P. Gogolak, E. Rajnavolgyi, and L. Nagy. 2009. Toll-like receptor 2-dependent induction of vitamin D3 receptor expression in dendritic cells. J. Immunol. 182: 5353–5361.
47. Dong, D., S. E. Ruuska, D. J. Levinthal, and N. Noy. 1999. Critical roles for cellular retinoic acid binding protein II and the retinoic acid-dependent nuclear receptor. Mol. Cell. Biol. 19: 7158–7167.

Dong, D., S. E. Ruuska, D. J. Levinthal, and N. Noy. 1999. Distinct roles for cellular retinoic acid-binding proteins I and II in regulating signaling by retinoic acid. J. Biol. Chem. 274: 23695–23698.
48. Nakken, B., T. Varga, I. Szatmari, L. Széles, A. Goyngosi, P. A. Illarionov, B. Dezso, P. Gogolak, E. Rajnavolgyi, and L. Nagy. 2011. Peroxisome proliferator-activated receptor gamma-regulated cathespisin D is required for lipid antigen presentation by dendritic cells. J. Immunol. 187: 240–247.
49. Elgueta, R., F. E. Sepulveda, F. Vilches, L. Vargas, J. R. Mora, M. R. Bono, and M. Rosenthal. 2008. Imprinting of CCR9 on CD4 T cells requires IL-4 signaling on mesenteric lymph node dendritic cells. J. Immunol. 180: 6501–6507.
50. Feng, T., Y. Cong, H. Qin, E. N. Benveniste, and C. O. Elson. 2010. Generation of mucosal dendritic cells from bone marrow reveals a critical role of retinoic acid. J. Immunol. 185: 5915–5922.
51. Iliev, I. D., I. Spadoni, E. Miletic, G. Matteoli, A. Sonzogni, G. M. Sampietro, D. Foschi, F. Caprioli, G. Viale, and M. Rescigno. 2009. Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. Gut. 58: 1481–1489.
52. Manicasamy, S., R. Ravindran, J. Deng, H. Ohlou, T. L. Denning, S. P. Kasturi, K. M. Rosenthal, B. D. Evavold, and B. Pulendran. 2009. Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity. Nat. Med. 15: 401–409.
53. Schug, T. T., D. C. Berry, N. S. Shaw, S. N. Travis, and N. Noy. 2007. Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. Cell. 129: 725–733.
54. Calabi, F. J., M. Jarvis, L. Martin, and C. Milstein. 1989. Two classes of CD1 genes. Eur. J. Immunol. 19: 285–292.
55. Kawano, T., J. Cui, Y. Koezuka, I. Taura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, et al. 1997. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science. 278: 1626–1629.
56. Adachi, M., R. Kurotani, K. Morimura, Y. Shah, M. Sanford, B. B. Madison, D. L. Gumucio, H. E. Marín, J. M. Peters, H. A. Young, et al. 2006. Peroxisome proliferator activated receptor gamma in colonic epithelial cells protects against experimental inflammatory bowel disease. Gut. 55: 1104–1113.
57. Bassaganya-Riera, J., K. Reynolds, S. Martino-Catt, Y. Cui, L. Hennighausen, F. Gonzalez, J. Rohrer, A. U. Benninghoff, and R. Hontecillas. 2004. Activation of PPAR gamma and delta by conjugated linoleic acid mediates protection from experimental inflammatory bowel disease. Gastroenterology. 127: 777–791.
58. Dubusquoy, L., E. A. Jansson, S. Deeb, S. Rakotobe, M. Karou, J. F. Colombel, J. Anwerx, S. Pettersson, and P. Desreumaux. 2003. Impaired expression of peroxisome proliferator-activated receptor gamma in ulcerative colitis. Gastroenterology. 124: 1265–1276.
59. Desreumaux, P., L. Dubusquoy, S. Nutten, M. Peuchmaur, W. Englaro, K. Schooijens, B. Derijard, B. Desvergne, W. Wahl, P. Chambon, et al. 2001. Attenuation of colon inflammation through activators of the retinoid X receptor (RXR)/peroxisome proliferator-activated receptor gamma (PPARgamma) heterodimer. A basis for new therapeutic strategies. J. Exp. Med. 193: 827–838.
60. Shah, Y. M., K. Morimura, and F. J. Gonzalez. 2007. Expression of peroxisome proliferator-activated receptor gamma in macrophage suppresses experimentally induced colitis. Am. J. Physiol. Gastrointest. Liver Physiol. 292: G657–G666.
61. Marcy, T. R., M. L. Britton, and S. M. Blevins. 2004. Second-generation thiazolidinediones and hepatotoxicity. Ann. Pharmacother. 38: 1419–1423.
62. Nesto, R. W., D. Bell, R. O. Bonow, V. Fonseca, S. M. Grundy, E. S. Horton, M. Le Winder, D. Porte, C. F. Semenkovich, S. Smith, et al. 2003. Thiazolidinedione use, fluid retention, and congestive heart failure: a consensus statement from the American Heart Association and American Diabetes Association. October 7, 2003. Circulation. 108: 2941–2948.
63. Bassaganya-Riera, J., M. DíGuardo, M. Climent, C. Vives, A. Carbo, Z. E. Jouni, A. W. Einerhand, M. O’Shea, and R. Hontecillas. 2011. Activation of PPARgamma and delta by dietary punicic acid ameliorates intestinal inflammation in mice. Br. J. Nutr. 106: 878–886.
64. Bassaganya-Riera, J., M. Villadomí, M. Pedragosa, C. De Simone, A. Carbo, R. Shakhshutdinov, C. Jobin, J. C. Arthur, B. A. Corl, H. Vogel, et al. 2012. Probiotic bacteria produce conjugated linoleic acid locally in the gut that targets macrophage PPAR gamma to suppress colitis. PLoS ONE. 7: e31238.
65. Lytle, C., T. J. Tod, K. T. Vo, J. W. Lee, R. D. Atkinson, and D. S. Strauss. 2005. The peroxisome proliferator-activated receptor gamma ligand rosiglitazone delays the onset of inflammatory bowel disease in mice with interleukin 10 deficiency. Inflamm. Bowel Dis. 11: 231–243.