BACKGROUND & AIMS: Gut-associated dendritic cells (DC) metabolize vitamin A into all-trans retinoic acid (RA), which is required to induce lymphocytes to localize to the gastrointestinal tract and promotes the differentiation of Foxp3+ regulatory T cells and IgA antibody–secreting cells. We investigated whether RA functions in a positive-feedback loop in DC to induce its own synthesis. METHODOLOGY: We measured levels of retinoids in intestinal tissues from mice and assessed the role of RA in the functional specialization of gut-associated DC in cell cultures and mice. We used pharmacologic antagonists to determine the signaling pathways involved in regulation of DC and used MyD88−/− mice to determine the contribution of Toll-like receptor signaling in RA-mediated effects on DC.

RESULTS: The concentration of retinoids decreased in a proximal-to-distal gradient along the intestine, which correlated with the activity of gut-specific DC. Importantly, RA regulated the ability of gut-associated DC to produce RA, induce T cells to localize to the gastrointestinal tract, and generate regulatory T cells and IgA-secreting cells. RA was sufficient to induce its own production by extraintestinal DC in vitro and in vivo. RA-mediated regulation of DC required signaling through the mitogen-activated protein kinase signaling pathway and unexpectedly required MyD88, which is conventionally associated with Toll-like receptor, interleukin-1, and interleukin-18 signaling. CONCLUSIONS: RA is necessary and sufficient to induce DC to regulate T-cell localization to the gastrointestinal tract and IgA secretion. Our findings also indicate crosstalk between the RA receptor and MyD88-dependent Toll-like receptor signaling pathways.

Keywords: Inflammation; IBD; Immune Response; Mouse Model.
Results
DC Ability to Imprint Gut Homing Correlates With Retinoid Levels in the Gut

RA and its precursors retinol and retinyl esters were readily detected in the intestinal mucosa, and their concentrations followed a gradient from proximal to distal, with the highest concentrations being found in the duodenum and jejunum (Figure 1A). Interestingly, retinoid levels correlated with DC imprinting ability. PP-DC and lamina propria DC (LP-DC) from duodenum and jejunum induced higher levels of gut-homing receptors (α4β7 and CCR9) and Foxp3+ T cells as compared to their counterparts in ileum, colon, or spleen (Figure 1B and C, Supplementary Figure 1A). Reciprocally, the skin-homing receptors E- and P-selectin ligands were more efficiently induced by ileum PP-DC/LP-DC, colon LP-DC, and spleen DC than by duodenum or jejunum PP-DC/LP-DC (Supplementary Figure 1B), consistent with the notion that induction of these receptors occurs as a default pathway in T cells activated without RA.7 Retinal dehydrogenases (RALDH) are critical enzymes for RA biosynthesis. Consistent with their higher gut-homing imprinting capacity, PP-DC and LP-DC from duodenum and jejunum exhibited higher RALDH activity (Aldefluor staining) than ileum or colon DC (Figure 1D), suggesting that Aldh1a2 mRNA levels in DC do not always fully correlate with their RALDH activity or gut-homing imprinting capacity. Whether this dissociation between Aldh1a2 mRNA and RALDH activity reflects local differences in RALDH protein expression or stability remains to be determined. In addition, using DR5-luciferase reporter mice, in which luciferase is controlled by a promoter with RA response elements,13 we determined that all tested DC have the capacity to respond to RA ex vivo (Supplementary Figure 1E). However, consistent with their exposure to high levels of RA, DC from the proximal small intestine exhibited higher luciferase activity than distal DC (Figure 1E).

RA Is Necessary in vivo for Gut-Associated DC Education

We depleted mice of the RA precursor retinol by feeding them a vitamin A–deficient (VAD) diet, as described.5,7 Because vitamin A is abundantly stored in the liver, it is difficult to attain complete vitamin A depletion, even when using a VAD diet for several months.14 To address this shortcoming, we used mice deficient in lecithin:retinol acyltransferase (LRAT), which cannot store retinol in the liver.14 LRAT-deficient mice develop normally when maintained on a vitamin A–sufficient diet, but they become vitamin A–depleted after only 2–4 weeks on a VAD diet, with the additional advantage of avoiding potential unwanted effects of chronic vitamin A depletion.14 In agreement with a critical role of RA in gut-associated DC education, PP-DC and MLN-DC from VAD mice induced lower levels of gut-homing receptors
on T cells as compared to their counterparts from mice on a vitamin A–sufficient diet (Figure 2A). In addition, MLN-DC and PP-DC from VAD mice were impaired in inducing Foxp3+ T cells (Figure 2B) and IgA-ASC (Figure 2C), respectively. Moreover, MLN-DC from VAD mice showed a marked reduction in Aldh1a2 mRNA expression (Figure 2D) and an impaired RALDH activity in total and CD11c+MLN-DC (Figure 2E), which was rescued by oral RA supplementation (Figure 2F). Thus, RA is necessary in vivo to educate gut-associated DC with critical gut-specific imprinting properties.

RA Is Sufficient to Confer DC With RA Synthesizing Capacity in vitro and in vivo

Murine spleen DC pretreated with RA (RA-DC) up-regulated Aldh1a2 mRNA (Figure 3A and Supplementary Figure 2A and B). This effect was not limited to RA, as other natural and synthetic RA receptor-retinoid X receptor (RAR-RXR) agonists (Am80, 9-cis RA, 13-cis RA) also induced Aldh1a2 in spleen-DC. In contrast, agonists for RXR (HX630, PA024), peroxisome proliferator-activated receptor (PPAR)-γ (rosiglitazone), PPAR-β/δ (GW0742) PXR (lithocholic acid), LXR (TO901317), or aryl hydrocarbon receptor (AHR) (ITE: 2-(1H-indole-3-carbonyl)-thiazole-4-carboxylic acid methyl ester) nuclear receptors did not induce Aldh1a2 mRNA. RA also induced Aldh1a2 in murine peripheral lymph node (PLN)-DC and bone marrow–derived DC (Supplementary Figure 2C), as well as ALDH1A2 mRNA in human monocyte-derived DC (Mo-DC) (Supplementary Figure 2D), suggesting that our findings could be extrapolated, at least in part, to human DC.

Consistent with their higher expression of Aldh1a2 mRNA, RA-DC displayed higher RALDH activity as compared to untreated spleen-DC (UT-DC) (Figure 3B), which was also observed when treating PLN-DC and bone marrow–DC with RA (Supplementary Figure 2E), indicating that RA-DC acquired RA-synthesizing capacity. Importantly, RA-DC induced significantly higher levels of gut-homing receptors α4β7 and CCR9 on activated T cells as compared to UT-DC, which was abrogated when T cells were activated in retinol-free media (Figure 3C) or in the presence of the RALDH inhibitor diethylaminobenzaldehyde (DEAB)17 (Figure 3D), implying that induction of gut-homing receptors by RA-DC required active RA synthesis and was not due to RA carry-over, which can happen when DC are incubated with high concentrations of RA.18 Similarly, human Mo-DC pretreated with RA induced higher levels of gut-homing receptors on polyclonally activated human T cells than untreated Mo-DC (Supplementary Figure 2F). Adding the RAR-inhibitor LE540 during the coculture abrogated gut-homing imprinting by RA-DC (Supplementary Figure 2G), indicating that gut-homing imprinting by RA-DC requires RA activity via RAR nuclear receptors. Consistent with their higher expression of gut-homing receptors, T cells activated with RA-DC migrated significantly more to the small intestine as com-
pared to those activated with UT-DC (Figure 3E). In addition, analogous to gut-associated DC, RA-DC also promoted the induction of Foxp3+ regulatory T cells (Figure 3F).

To obtain mechanistic clues on Aldh1a2 induction by RA in DC, we incubated spleen-DC from DR5-luciferase mice with RA in the presence or absence of the transcription inhibitor actinomycin-D or the translation inhibitor cycloheximide as described. RA-treated spleen-DC exhibited increased RALDH and luciferase activities (induction of Aldh1a2 mRNA and a RAR-dependent reporter by RA, respectively), which were abrogated by actinomycin-D or cycloheximide treatment (Supplementary Figure 3A and B), suggesting that RA-mediated DC education to express RALDH requires transcription and protein synthesis. Moreover, actinomycin-D inhibited RA-mediated induction of Aldh1a2 mRNA and Rarb mRNA (encoding RARβ, a known RA-induced gene) (Supplementary Figure 3C), indicating that their increase is mostly due to de novo mRNA stability. Interestingly, cycloheximide blocked Aldh1a2 but not Rarb mRNA induction, suggesting that the increased Aldh1a2 mRNA transcription in RA-treated spleen-DC depends on newly synthesized proteins, whereas Rarb appears to be directly induced by RA without requiring prior protein synthesis. This finding is consistent with their distinct transcription kinetics, as Rarb mRNA was clearly induced as quickly as 1 hour post-RA treatment, whereas Aldh1a2 mRNA exhibited a more delayed kinetics (Supplementary Figure 3D). In sum, RA induction of Aldh1a2 mRNA in spleen-DC appears to occur indirectly in a process that requires prior de novo protein synthesis.

Of note, although spleen-DC acquired RALDH activity when pretreated with RA for 24 hours, this was not observed when DC were acutely exposed to RA for 30 minutes (ie, only during the Aldefluor assay) (Supplementary Figure 3E). Moreover, MLN-DC maintained the same high RALDH activity regardless of whether the mice were supplemented with oral RA (Supplementary Figure 3F). Thus, RA does not appear to directly induce, enhance, or otherwise affect RALDH enzymatic activity, suggesting that its main effect on DC education involves Aldh1a2 mRNA induction and de novo RALDH protein synthesis.

To assess whether RA can also educate extraintestinal DC in vivo, we treated mice with RA via oral gavage and then analyzed PLN-DC for their expression of Aldh1a2 mRNA, RALDH activity, and gut-homing imprinting capacity (Figure 4A). As expected, PLN-DC from control mice showed much lower levels of Aldh1a2 mRNA and RALDH activity as compared to MLN-DC (Figure 4C), indicating that their increase is mostly due to de novo transcription rather than enhanced mRNA stability. Interestingly, cycloheximide blocked Aldh1a2 but not Rarb mRNA induction, suggesting that the increased Aldh1a2 mRNA transcription in RA-treated spleen-DC depends on newly synthesized proteins, whereas Rarb appears to be directly induced by RA without requiring prior protein synthesis. This finding is consistent with their distinct transcription kinetics, as Rarb mRNA was clearly induced as quickly as 1 hour post-RA treatment, whereas Aldh1a2 mRNA exhibited a more delayed kinetics (Supplementary Figure 3D). In sum, RA induction of Aldh1a2 mRNA in spleen-DC appears to occur indirectly in a process that requires prior de novo protein synthesis.

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on the presence of retinol and RALDH enzymatic activity (Figure 4D), demonstrating that it required active RA biosynthesis.

We could not detect RA in PLN from untreated animals, even using the most sensitive methods;\(^1\) whereas RA was readily detected in PLN from RA-treated animals with levels approaching those found in MLN from untreated animals (Supplementary Figure 4B). Moreover, luciferase activity in PLN cells from RA-treated DR5-luciferase mice was similar to that observed in MLN or PP cells from control animals (Supplementary Figure 4C). Of note, RA supplementation was associated with increased proportions of activated CCR9\(^+\) CD8 T cells in PLN and spleen upon subcutaneous immunization, reaching levels comparable to control MLN and PP cells from control animals (Supplementary Figure 4C).

In summary, our data show that RA is sufficient to induce a positive feedback loop in extraintestinal DC, conferring them with the capacity to synthesize RA and to generate gut-homing T cells in vitro and in vivo.

### RA-Mediated DC Education Requires Extracellular Signal-Regulated Kinase (ERK)/Mitogen-Activated Protein Kinase (MAPK) Signaling

To obtain further mechanistic insights on how RA educates DC, we tested the role of canonical signaling pathways in RA-mediated DC education by adding pharmacological inhibitors concomitant with RA treatment. Blockade of p38/MAPK (SB203580), c-Jun-N-terminal kinase/MAPK (SP600125), or nuclear factor-κB (SN50) pathways\(^3\) did not affect RA-mediated DC education (Figure 5A). However, inhibition of ERK/MAPK (U0126)\(^3\) during RA treatment of DC significantly decreased their capacity to induce gut-homing receptors on T cells. Moreover, blocking ERK completely abolished Aldh1a2 mRNA induction in RA-DC (Figure 5B), indicating that ERK signaling is required for RA-mediated DC education. Interestingly, ERK inhibition in spleen-DC isolated from DR5-luciferase mice abrogated RA-mediated luciferase induction (Figure 5C), suggesting that ERK signaling might be required for general RAR-dependent effects on DC.

To assess whether ERK signaling plays a physiological role in gut-associated DC education, we treated mice with PD0325901, an orally bioavailable ERK inhibitor that has...
been used in vivo. Consistent with a physiological role of ERK signaling in gut-associated DC education, treatment with PD0325901 significantly decreased RALDH activity in endogenous CD103+ MLN-DC (Figure 5D), without decreasing the frequency of CD103+ DC in MLN (data not shown).

Thus, ERK signaling is needed for RA-mediated DC education and is also required in vivo for endowing gut-associated DC with RA-synthesizing capacity.

**MyD88 Is Required for RA-Mediated DC Education**

Given that some TLR agonists can induce RALDH enzymes in DC in an ERK-dependent manner and that RA-mediated DC education also requires ERK signaling, we explored whether TLR signals might modulate RA effects on DC. Consistent with this possibility, spleen-DC from mice lacking MyD88 (essential for most TLR signaling) were impaired in their RA-mediated DC education. MyD88−/− RA-DC induced lower levels of gut-homing receptors on T cells as compared to wild-type RA-DC (Figure 6A), and the induction of Aldh1a2 mRNA was completely abrogated in MyD88−/− RA-DC (Figure 6B). Similarly, Tgm2 mRNA (encoding tissue transglutaminase, a well-known RA target gene) was not induced in MyD88−/− RA-DC, whereas it was readily induced in wild-type RA-DC (Figure 6B), suggesting that MyD88 might fulfill a more general role in RA-mediated effects on DC. Of note, DC subset composition and maturation status were comparable in MyD88−/− and wild-type spleen-DC (data not shown).

Next, we asked whether MyD88 was required in vivo for RA-mediated DC education. MyD88−/− or wild-type mice were orally supplemented with RA. PLN-DC isolated from RA-treated MyD88−/− mice exhibited lower RALDH activity than PLN-DC from RA-treated wild-type mice (Figure 6C and Supplementary Figure 5A). Moreover, spleen-DC from RA-treated MyD88−/− mice induced lower levels of α4β7 and CCR9 on T cells as compared to spleen-DC from RA-treated wild-type mice (Supplementary Figure 5B). In addition, the expression of the RA-induced gene Tgm2 was lower in MyD88−/− PP-DC as compared to wild-type PP-DC, whereas Fabp4 mRNA, a PPARγ-RXR target gene, was expressed at similar levels in MyD88−/− and wild-type PP-DC (Supplementary Figure 5C). These data suggest that MyD88 expression is necessary for RAR-RXR−mediated effects on DC, but is not generally required for the activity of other nuclear receptors sharing the RXR nuclear receptor partner.

Wild-type and MyD88−/− spleen-DC did not differ in the expression of CrabpII and Fabp5 mRNA, which encode proteins that modulate RA signaling by channeling RA to either RAR-RXR or PPARβ/δ-RXR, respectively (Supplementary Figure 5D).
Interestingly, RA induced Tlr1 and Tlr2 mRNA (Supplementary Figure 5E). Thus, RA might contribute to sensitizing DC to TLR1/2 ligands, suggesting a potential intersection point between TLR- and RA-mediated effects on DC. In addition, RA induced Rarb mRNA in DC (Supplementary Figure 3C and D, Supplementary Figure 5E), hence exerting a positive feedback loop in its signaling.

We found that RA-mediated education was ERK-dependent (Figure 5), and it has been shown that TLR engagement results in ERK activation in DC.20 Moreover, RA has been shown to rapidly induce ERK phosphorylation,23 suggesting that ERK signaling might represent another potential intersection point between TLR- and RA-mediated signaling in DC. Therefore, we assessed whether RA can induce ERK phosphorylation in spleen-DC from wild-type and MyD88−/− mice. Interestingly, although RA induced ERK1/2 phosphorylation in spleen-DC, this effect was decreased on MyD88−/− DC (Supplementary Figure 5F and G). ERK phosphorylation was not affected in TLR4−/− spleen-DC, hence, excluding an effect due to lipopolysaccharide (LPS) contamination. These results suggest that RA induces ERK1/2 phosphorylation, an effect that requires MyD88 expression in spleen-DC.

The mechanism by which lack of MyD88 impairs RA-mediated ERK1/2 phosphorylation remains undefined, although we hypothesize that it might be related, at least in part, to decreased RA-mediated signaling. In fact, whereas wild-type and MyD88−/− spleen-DC did not differ in the expression of most RAR and RXR isoforms, Rarb mRNA (encoding RARβ) was not detected in MyD88−/− spleen-DC (Figure 6D), suggesting that lack of RARβ might explain, at least in part, the decreased RA-mediated education on MyD88−/− DC. In agreement with this possibility, LE540, a predominantly RARβ inhibitor,24 completely abrogated RA-mediated Aldh1a2 induction (Figure 6E) and RA-induced luciferase activity in spleen-DC from DR5-luciferase mice (Figure 6F). Thus, the mechanism by which MyD88 controls RA-mediated effects on DC might involve RARβ modulation.

**TLR Signals Contribute to RA-Mediated DC Education**

Because our data suggest that MyD88 is critical for RA-mediated DC education, we hypothesized that TLR agonists contribute to DC education. Consistent with this possibility, human Mo-DC treated with RA and/or a
TLR1/2-agonist (Pam3CSK4) exhibited higher RALDH activity as compared to untreated Mo-DC (Figure 7A). The increased RALDH activity in Mo-DC correlated with their ability to induce Foxp3 in human CD4 T cells (Figure 7B). Moreover, mouse spleen-DC or human Mo-DC treated with RA plus Pam3CSK4 induced higher proportions of α4β7+ and CCR9+ T cells as compared to DC treated with either RA or Pam3CSK4 alone (Figure 7C and D). Furthermore, TLR1/2 stimulation and RA synergized to confer spleen-DC with the capacity to induce IgA-ASC upon B-cell activation (Figure 7E), an effect that correlated with the up-regulation of April, Baff, and Nos2 in DC (encoding APRIL:a proliferation-inducing ligand, BAFF:B-cell-activating factor, and inducible nitric oxide synthase, respectively) (Supplementary Figure 6), all of which are important factors for inducing IgA-ASC.25 Of note, RA-treated human Mo-DC did not enhance IgA production by polyclonally activated human B cells (data not shown), suggesting that Mo-DC (differentiated with IL-4 and granulocyte-macrophage colony-stimulating factor) are not permissive for IgA class-switching or that there are some species-specific differences in this regard. Therefore, in addition to modulating RA synthesis and gut-homing imprinting, RA- and MyD88-dependent pathways synergize to confer DC with the capacity to induce Foxp3 T cells and IgA-ASC, which are hallmarks of mucosal-specific imprinting.

**Discussion**

RA is synthesized from all-trans retinol, which is obtained from the food as all-trans retinol, retinyl esters, or β-carotenes (provitamin A).26 Interestingly, we found that retinoids follow a proximal-to-distal gradient in the intestine, with the highest levels being found in the duodenum and jejunum. This retinoid gradient might be explained, at least in part, by differences in the absorption and/or synthesis of retinoids along the intestinal tract, which is supported by the higher levels of carotenoid receptor scavenger receptor type B, class I and carotenoid metabolizing enzyme β,β-carotene-15,15′-monoxygenase 1 found in the proximal intestine as compared to ileum.27 A similar proximal-to-distal expression gradient has been described for cellular retinol binding protein—II
and LRAT,\textsuperscript{28} which might contribute to further enhancing the pool of retinol and retinyl esters found in the proximal intestine. Of note, the CCR9-ligand CCL25 also shows a proximal-to-distal gradient\textsuperscript{29} (Supplementary Text 1).

A model in which RA is physiologically required to induce its own synthesis implies the existence of another (primary) source of RA to educate DC. It has been shown that intestinal epithelial cells (IECs) express high levels of RALDH\textsuperscript{10,31} and can produce RA.\textsuperscript{7,32} In fact, in contrast to RALDH2, which is decreased in MLN-DC from VAD mice (our data and reference 17), RALDH1 expression in IEC does not decrease in VAD mice,\textsuperscript{32,33} suggesting that IEC might be “hard-wired” to synthesize RA or that their RA-synthesizing capacity depends on other environmental factors (eg, TLR signals, microbiota). In summary, IEC might provide, at least in part, a primary supply of RA in the gut mucosa, which in turn induces RALDH2 in gut-associated DC, conferring them with RA-synthesizing capacity (Supplementary Text 2).

A recent report suggested that GM-CSF is physiologically required for gut-associated DC education and that RA might be important for DC education by inducing GM-CSF-expressing macrophages in MLN.\textsuperscript{17} However, we did not observe impairment in Aldh1a2 mRNA expression, RALDH activity, or gut-homing imprinting capacity in gut-associated DC from GM-CSF\textsuperscript{-/-} mice (Wang et al, J Immunol, in press), suggesting that GM-CSF might not be essential for gut-associated DC education in vivo. These discrepancies could be partially explained by the fact that we used DC from mice deficient only in GM-CSF, whereas Yokota et al isolated DC from mice deficient in the common $\beta$-c subunit, which is shared by GM-CSF, IL-3, and IL-5 receptors.\textsuperscript{17}

Similar to TLR2 stimulation,\textsuperscript{30} RA-mediated DC education required signaling via ERK/MAPK. This finding is in line with previous studies showing that some RA-mediated effects depend on ERK/MAPK, but not on p38/MAPK or c-Jun-N-terminal kinase/MAPK signaling.\textsuperscript{34} However, despite the fact that RA share some signaling mechanisms with TLR2 stimulation, including ERK/MAPK and MyD88, these pathways do not appear to be redundant because we observed additive and even synergistic effects on DC education when pretreating DC with RA and a TLR1/2-agonist (see also Supplementary Text 3).

We show that RA is a critical factor in gut-associated DC education, which is necessary and sufficient to confer DC with key gut-synthesizing imprinting properties, including the capacity to synthesize RA, imprint gut-tropic lymphocytes and regulatory T cells, as well as promote IgA-ASC differentiation (Supplementary Figure 7). Nevertheless, some of our results in murine DC were not observed in human Mo-DC, and further investigation will be needed to determine if there are some species-specific differences in this regard.

In conclusion, our data highlight an unexpected link between RAR nuclear receptor and MyD88/TLR-dependent pathways, indicating that gut-associated DC special-

### Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at doi:10.1053/j.gastro.2011.04.010.

### References


Fig. S1. (A-B) CD8 T cell were activated with Sp-DC, PP-DC from duodenum (duo), jejunum (jej), ileum (ile) or colon LP-DC and analyzed after 4 days. (A) Expression of Ccr9 mRNA in activated T cells. As positive control, 10 nM RA was supplemented to some co-cultures with Sp-DC. Graph is representative of two independent experiments. (B) Expression of P- and E-selectin ligands on activated T cells (n=5). (C) CD103 expression in LP-DC from duo, ile and colon. (D) Aldh1a2 mRNA expression in PP-DC from duo, jej, ile and LP-DC from colon. Sp-DC and MLN-DC are shown as a reference (n=6). (E) DC from different tissues were isolated from DR5-luciferase mice and incubated for 24 h with or without 10 nM RA. After that, they were analyzed for their luciferase activity (n=2). Mean ± SEM, *p<0.05
**Supplementary Figure 2**

**A**

![Graph A](image)

**B**

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**C**

![Graph C](image)

**D**

![Graph D](image)

**E**

![Table E](image)

**F**

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**G**

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**Fig. S2.** (A-B) Sp-DC were treated with (RA-DC) or without (UT-DC) 100 nM RA. (A) Kinetics for Aldh1a2 mRNA expression and (B) pooled data at 24 h (n=7). (C) Aldh1a2 mRNA levels on Sp-DC, PLN-DC or BM-DC treated or not with RA (100 nM). (D) Human monocytes were differentiated to DC (Mo-DC) and at day 6 were incubated for 24 hours with (RA) or without (UT) 100 nM RA. ALDH1A2 mRNA expression in Mo-DC normalized respect to GAPDH mRNA (n=4). (E) PLN-DC or differentiated Bone marrooe-derived DC (BM-DC) were treated or not with different concentration of RA. Upon 24 h, RALDH activity was measured using Aldefluor on CD11c cells. One representative experiment out of 2. (F) Human monocytes were differentiated as in (D). UT or RA Mo-DC were co-cultured with total human T cells activated with plate-bound anti-CD3 plus anti-CD28 antibodies. After 6 days, CD4 and CD8 T cells were analyzed for their expression of α4β7 (n=5). Mean ± SEM, *p<0.05 (G) RA-DC were co-culture with CFSE-labeled naive P14 T cells in the presence or absence of 1 μM LE540. After 4 days, CD8 T cells were analyzed for their expression of α4β7.
Fig. S3. (A-D) Spleen-DC from DR5-luciferase mice were treated or not with 100 nM RA and in the presence or absence of the transcription inhibitor actinomycin-D (1 μg/ml) or the translation inhibitor cycloheximide (10 μg/ml). After 20 hours the cells were assessed for their Raldh activity (Aldefluor staining) (A) and luciferase activity (B). Aldh1a2 and Rarb mRNA were analyzed at either 5 hours (C) or at different time points (D). Mean ± range, (n=2).

(E) Raldh activity in spleen-DC treated or not with RA for either 24 hours or 30 min (i.e., only during the Aldefluor assay). (F) Raldh activity in MLN-DC or PLN-DC from mice untreated or treated orally with RA (400 μg/day for 5 days).
Fig. S4. (A) Wild type mice were orally treated with different concentration of RA (10-400 µg/day) for 4 days. PLN-DC and MLN-DC were isolated at day 4 and analyzed for their expression of Aldh1a2 mRNA. (B-C) Wild type mice (B) or DR5-luciferase mice (B) were treated with 400 µg/day RA or vehicle for 4 days (n=5 mice/group). (A) RA levels in PLN and MLN. (B) Luciferase activity in MLN, PP and PLN from DR5-luciferase mice. (D) Wild type Thy1.1+ congenic mice were treated with RA (400 µg/day for 3 days) or vehicle (olive oil) every day via oral gavage. At day 0 the mice were adoptively transferred with CFSE-labeled OT-I CD8 cells. At day 1 the mice were immunized with OVA (500 µg) + LPS (50 µg) s.c. After 3 days PLN, MLN and spleen were analyzed for CCR9 expression on proliferating Thy1.2+ CD8 T cells. (n=2-3). Mean ± SEM
Supplementary Figure 5

Fig. S5. (A) Wild type or MyD88−/− mice were supplemented orally with RA (400 µg/dose) every other day for 6 days. After that, PLN-DC were analyzed for Raldh activity (n=7). (B) Sp-DC from RA-supplemented wild type or MyD88−/− mice were used to activate naïve CD8 T cells. After 4 days T cells were analyzed for α4β7 and CCR9 expression. FACS plots show one experiment using DC pooled from 4 mice. (C) PP-DC from wild type or MyD88−/− mice were analyzed for their expression of Tgm2 and Fabp4 mRNA (n=7). (D) Spleen-DC from wild type or MyD88−/− mice were analyzed for their expression of Crabp2 and Fabp5 mRNA. (E) Kinetics of Tlr1, Tlr2 and Rarb mRNA expression in spleen-DC upon RA or Pam3CSK4 treatment (n=3). Mean ± SEM. (F) Wild type, MyD88−/− and TLR4−/− spleen-DC were treated with RA for 0, 30, 60 and 90 minutes. Cell lysates were analyzed by Western blot for ERK1/2 phosphorylation and total ERK1/2. (G) Semi-quantitative analysis of p-ERK1/2 and total ERK1/2 normalized versus untreated samples (n=2). Mean ± SEM. *p<0.05
Fig. S6. Sp-DC were incubated for 24 h with or without 100 nM of RA, Pam3CSK4 (0.5 μg/ml) or both and then analyzed for their expression of *April, Baff* and *Nos2* mRNA (n=3).
**Fig. S7. Model for RA-mediated DC education.** RA acts on DC via RAR-RXR nuclear receptors to induce Aldh1a2 expression (encoding Radh2). Raldh2 metabolizes retinal into RA, which induces gut-homing receptors on T cells. In addition, RA potentiates the differentiation of IgA-ASC and Foxp3\(^+\) T\(_{REG}\). RA also induces Nos2 (encoding iNOS) in DC, hence boosting the induction of IgA-ASC by increasing nitric oxide (NO). MyD88 is required for RA-mediated DC education, probably by modulating Rarb expression (encoding RAR\(\beta\)) and/or by activating ERK/MAPK, which is also needed for RA-mediated effects on DC.
Supplemental Text-1

Consistent with a physiological role of RA in DC education, the proximal-to-distal retinoid gradient tightly correlated with DC imprinting abilities. DC from duodenum and jejunum induced higher levels of gut-tropic and Foxp3+ T cells as compared to DC from distal intestinal segments. Since CCL25 (CCR9-ligand) also follows a proximal-to-distal gradient \(^1\), it is tempting to speculate that DC from duodenum/jejunum might be preferentially involved in the establishment of oral tolerance by transporting food-borne antigens to the MLN where they would induce Foxp3’CCR9’ T\(_{REG}\) that will preferentially migrate to proximal intestinal segments, hence preventing undesired immune responses to innocuous antigens as soon as they have access to the intestinal mucosa.

Supplemental Text-2

In support of this possibility, it has been described that an IEC line promotes gut-homing imprinting when co-cultured with T cells activated with extra-intestinal DC \(^2\). Another study showed that IEC can condition extra-intestinal DC to induce T\(_{REG}\) \textit{in vitro}, an effect that was partially dependent on RA and TGF-\(\beta\) \(^3\). However, since there are no studies assessing the specific contribution of IEC-derived RA in gut-associated DC education \textit{in vivo}, the physiological relevance of IEC in this context remains to be determined.

Although our data show that RA readily upregulates \textit{Aldh1a2} mRNA (encoding Raldh2), RA did not consistently induce \textit{Aldh1a1} mRNA (encoding Raldh1) in extra-intestinal DC (data not shown), suggesting that the induction of Raldh1 and Raldh2 isozymes is differentially regulated. These considerations notwithstanding, it has been reported that gut-associated DC mainly express Raldh2 \(^4\) and that this isozyme is about 5-10 times more efficient at synthesizing RA as compared to Raldh1 \(^5\).

In addition, although RA has been shown to induce enzymes involved in the first step of its biosynthesis from retinol to retinal \(^6\), other studies have shown that it actually inhibits the expression of
Raldh enzymes in non-immune cells (including IEC), thus blocking the final step in the synthesis of RA \(^{7-10}\). By contrast, our data clearly show that RA induces a positive feedback loop in DC, which is additionally translated in functional effects on lymphocyte imprinting. Thus, whether RA promotes a positive or a negative feedback loop on its own synthesis seems to depend, at least in part, on the specific Raldh isoform and on the particular tissue/cell type analyzed.

**Supplemental Text-3**

Whereas the exact mechanism of how MyD88 controls RA effects on DC remains to be fully clarified, our results indicate that RARβ might be involved in RA-mediated DC education and that the expression of RARβ depends on MyD88 signaling. Of note, RARβ is also expressed in gut-associated follicular DC (FDC) and its expression depends on RA \(^{11}\), suggesting that RA also modulates the expression of this RAR isoform.

Besides modulating DC to synthesize RA and to induce gut-tropic T cells, RA was also required *in vivo* to confer gut-associated DC with IgA-inducing capacity and it synergized with TLR stimulation to confer extra-intestinal DC with IgA-inducing potential. It is likely that RA might influence DC via different mechanisms, including RA production itself, which can directly acts on B cells to promote differentiation of IgA-ASC \(^{12}\). In addition, RA can induce TGFβ synthesis by DC \(^{13}\) and we found that TLR1/2 stimulation or RA can induce Nos2 mRNA (encoding iNOS) in DC, which might additionally contribute to the induction of IgA-ASC \(^{14}\). In line with our observations, it has been described that RA can potentiate iNOS induction *in vivo* \(^{15}\).
Materials & Methods

Mice

OT-1xRAG2\(^{-/-}\), P14xTCR\(\alpha\)/\(\beta\), C57BL/6, and C57BL/6/Thy1.1\(^+\) mice were purchased from Taconic (Germantown, NY). MyD88\(^{-/-}\) mice\(^{16}\) were provided by Dr. Nir Hacohen (Massachusetts General Hospital, Boston, MA). DR5-luciferase mice\(^{17}\) were provided by Dr. Rune Blomhoff (Cgene AS, Oslo, Norway). LRAT\(^{-/-}\) mice\(^{18}\) were provided by Dr. William Blaner (Columbia University, NY, USA). Mice were maintained in SPF/VAF animal facilities at Massachusetts General Hospital (MGH) and used in accordance with the guidelines from the Subcommittee on Research Animal Care at MGH and Harvard Medical School.

Reagents

Nuclear receptor agonists: Pan-RAR-agonist all-\(trans\) RA, Pan-RAR/Pan-RXR-agonist 9-\(cis\) RA, Pan-RAR-agonist 13-cis RA and the PXR-agonist Lithocholic acid, actinomycin-D and cycloheximide were purchased from Sigma (St Louis, MO); LXR-agonist TO901317 (Cayman, Ann Arbor, MI); PPAR\(\beta/\delta\)-agonist GW0742 (Tocris, Ellisville, MI); AHR-agonist 2-(19H-indole-39-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) (Tocris, Ellisville, MI); PPAR\(\gamma\)-agonist Rosiglitazone (Cayman, Ann Arbor, MI); RXR-agonists PA024 and HX630; and RAR\(\alpha/\beta\)-agonist Am80 were provided by Dr. Hiroyuki Kagechika (Tokyo Medical and Dental University, Japan); Raldh inhibitor 4-(diethylamino)-benzaldehyde (DEAB) (Stemcell, Vancouver); RAR\(\beta\)-antagonist LE540 (Wako Chemicals USA, Richmond, VA) was dissolved in DMSO or ethanol (1 mM stocks) and used at 1 \(\mu\)M final concentration. The following mAb used to label murine cells were purchased from BD Biosciences (San Jose, CA): anti-\(\alpha\)4b7 (DATK32), anti-B220 (RA3-6B2), anti-CD3 (17A2), anti-CD19 (1D3), anti-Pan-NK (DX5), anti-Ly-76 (Ter-119), anti-CD11c (HL3), anti-CD103 (M290) and
anti-IgA (C10-1). Anti-mouse CCR9 (CW-1.2) was from eBioscience (San Diego, CA). MAb used to label human cells: anti-CD4 (clone SK3) and anti-CD8 (clone SK1) were from BD Biosciences (San Jose, California). Anti human-α4β7 (ACT-1) was from Millennium Pharmaceuticals (Cambridge, MA). TLR1/2 ligand (Pam3CSK4) was from InvivoGene (San Diego, CA). Pharmacological inhibitors for P38/MAPK (SB203580), ERK1/2/MAPK (U0126), JNK/MAPK (SP600125) and NF-κB (SN50) were from Calbiochem (EMD Biosciences, San Diego, CA) and have been described. The ERK1/2-inhibitor PD0325901 used for in vivo experiments was purchased from Stemgent (Cambridge, MA). The cell tracers CFSE (Carboxyfluorescein diacetate, succinimidyl ester) and CMTMR (5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine) were from Molecular Probes (Invitrogen, USA). LCMVgp33–41 and ovalbumin SIINFEKL peptide were purchased from New England Peptides (Invitrogen, USA).

**Mouse DC isolation and conditioning**

C57BL/6 mice were injected subcutaneously with B16 melanoma cells secreting Flt3-L. After 12–17 days, the mice were euthanized and single-cell suspensions were generated by digestion with Liberase® TL (0.15mg/ml, Roche, Indianopolis, USA) and DNase 1 (325 Units/ml, Sigma-Aldrich) dissolved in HBSS medium without serum. DCs were immunomagnetically isolated by a first round of negative selection using MAb to CD3, CD19, Pan-and NK, and goat anti-rat IgG microbeads (Miltenyi Biotec) and a second round of positive selection using CD11c magnetic beads (Miltenyi Biotec). DC (>98% CD11c+) were treated for 24 hours with the indicated ligands, washed and used for co-culture with T and/or B cells, Aldefluor staining or RNA extraction. For co-culture with naïve CD8 T cells from TCR transgenic P14xTCRα−/− or OT-1xRAG2−/− mice, DC were pulsed for 2 h with 100 nM LCMVgp33–41 or ovalbumin SIINFEKL peptide, respectively, washed and used immediately.
**Lymphocyte/DC isolation and cocultures**

DC and naïve T cells were isolated and co-cultured as described. Naïve P14 CD8 T cells were purified from splenocytes after RBC lysis in ACK buffer by negative selection, using mAbs to B220, I-A/I-E (2G9), CD4 (H129.19), CD19, Pan-NK, Ly-76 (Ter-119), and Ly-6G (Gr-1) followed by goat anti-rat IgG microbeads. 1x10⁵ CFSE-labeled naïve T cells were cocultured with peptide-pulsed DC in a 1:1 ratio in flat bottom 96-well plates (Falcon, BD Biosciences). For CFSE labeling, T cells were resuspended at 10⁷ cells/ml in DMEM + 1% FBS + 20 mM Hepes, incubated with 2.5 mM CFSE for 20 min at 37°C and then washed using an FBS gradient.

Naïve B cells were purified from spleens by negative selection using anti-CD43 microbeads (Miltenyi Biotec, >90% B220⁺ cells). 1x10⁵ B cells were activated with 10 μg/ml anti-mouse IgM (Jackson Immunoresearch, USA) plus IL-5 (5ng/ml) either alone or plus Spleen-DC pre-treated or not with 100 nM RA (1:1 B cell:DC ratio). 4-5 days later, activated B220⁺ B cells/ plasmablasts were analyzed by flow cytometry for intracellular IgA expression and for IgA in the culture supernatant.

**In vivo RA and PD0325901 treatment**

C57BL/6 mice were treated with RA (400 μg/dose) or vehicle (olive oil) by oral gavage three times every other day. PD0325901 was dissolved in DMSO (50 mg/ml stock) and then diluted in water containing 0.05% hydroxypropyl-methylcellulose and 0.02% Tween 80 (final concentration: 2.5 mg/ml PD0325901). 250 μl/dose (25 mg/kg) were administered by oral gavage twice (day 0 and 3). Animals treated with either RA or PD0325901 were sacrificed one day after the last dose and CD11c⁺ positive cells were purified from MLN, PLN and spleen by using magnetic beads (>96% CD11c⁺). Raldh activity and/or mRNA levels were measured in the isolated DC by using Aldefluor® assay or TaqMan qPCR, respectively.
**Retinoid measurements**

Retinoid concentrations were assessed as described \(^{22}\). Briefly, tissue samples were homogenized in ground glass homogenizers (Kontes, size 22) in 0.5 to 1.0 mL saline (0.9% NaCl). All-trans RA was quantified by LC/MS/MS with APCI in positive ion mode on an API-4000 (Applied Biosystems). Retinol and retinyl esters were quantified by HPLC/UV on an Alliance 2690 (Waters). Retinoids in tissues are expressed as mol/g tissue.

**Human DC differentiation and T cell co-culture**

PBMCs from healthy donors (5-6\(\times\)10\(^7\)) were cultured in RPMI 3% human serum for 1 h at 37°C. Adherent cells were then cultured in RPMI 10% FBS supplemented with GM-CSF (100 ng/ml; R& D Systems, Lauderdale, MN, USA). At day 6, differentiated (Mo-DC) were collected and conditioned with or without RA (100 nM, Sigma). At day 7, DC were analyzed by FACS, washed for co-culture or processed for RNA extraction. In some experiments, Mo-DC were co-cultured with enriched allogeneic CFSE-labeled T cells (Pan T cell isolation kit II, Miltenyi Biotec) in a 1:1 T:DC ratio and T cells were activated using plate-bound anti-CD3 (OKT3, eBioscience) plus anti-CD28 (CD28.6, eBioscience) (10 μg/ml each). After 5-7 days T cells were collected, stained for CD4, CD8 and α4β7 and then analyzed by FACS. In Foxp3 induction experiments Mo-DC were co-culture with allogeneic CD4 T cells (human CD4 isolation kit, Miltenyi Biotec) activated with anti-human CD3 (10 μg/ml), anti-human CD28 (1 μg/ml) and hTGF-β1 (2 ng/ml) for 4 days.

**ELISA for IgA**

Measurements of IgA in the culture supernatants was performed as described \(^{12}\) using the ELISA Quantitation Kit for mouse and human IgA (Bethyl Laboratories, Montgomery, TX) according to the manufacturer’s protocol.
**Intracellular IgA labeling**

Activated B cells were labeled with anti-B220 (RA3-6b2) and CD138 (281-2). After that, they were fixed and permeabilized using the CytoFix/CytoPerm kit (BD Biosciences, USA), labeled intracellularly with anti-IgA (C10-1) or a matched isotype control and analyzed by flow cytometry \(^2\).

**Aldefluor staining**

Raldh activity was determined using the Aldefluor\(^®\) assay (StemCell technology, Vancouver, Canada), as described \(^4\). Briefly, cells suspension (1x10\(^6\) cells/ml) were incubated for 45 minutes at 37°C in Aldefluor assay buffer containing activated Aldefluor substrate in the presence or absence of Raldh inhibitor diethylaminobenzaldehyde (DEAB). The cells were subsequently stained with specific antibodies, washed, resuspended in Aldefluor assay buffer and analyzed in a FACScalibur (BD Biosciences).

**Quantitative PCR**

Total RNA was isolated by using RNeasy (Qiagen) and then retrotranscribed with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative PCR was performed using TaqMan PCR master mix (Applied Biosystems, Framingham, MA) using the following primers probes; *Aldh1a1* (Mm00657317_m1), *Aldh1a2* (Mm00501306_m1), *Rara* (Mm00436264_m1), *Rarb* (Mm01319674_m1), *Rarg* (Mm00441091_m1), *Rxra* (Mm00441182_m1), *Rxrb* (Mm00441193_m1), *Rxrg* (Mm00801693_g1), *Tgm2* (Mm00436980_m1), *Fabp5* (Mm00783731_s1), *CrabpII* (Mm00436410_m1), *Nos2* (Mm 01309901.m1) and *Actb* (NM_007393.1). For SYBR green we used the following primers: *Baff*-F: 5'AGG CTG GAA GAA GGA GAT GAG, *Baff*-R: 5’ CAG AGA AGA CGA GGG AAG GG, *April*-F: 5’ GGG GAA GGA GGA GTG TCA GAG TG, *April*-R: GCA GGG AGG GTG GGA ATA C, *Tgfb1*-F: 5’ TGG AGC AAC ATG TGG AAC TC, *Tgfb1*-R: 5’ TGC CGT CGT ACA
ACT CCA GTG AC, GAPDH-F: 5’ CAT GGC CTT CCG TGT TCC TA, GAPDH-F: 5’ GCG GCA CGT CAG ATC CA, Human ALDH1A2-F: 5’ GGG CAG TTC TTG CAA CCA TGG AAT Human ALDH1A2-F: 5’ TTT GAT GAC GCC CTG CAA ATC CAC, Human ACTB-F: AGG CCA ACC GCG AGA AGA TGA C Human ACTB-R: AGG TCC AGA CGC AGG ATG GCA T. The comparative Ct method was used to quantify transcripts that were normalized respect to Actb or GAPDH.

**Western blot**

Spleen-DC from wild type, MyD88⁻/⁻ or TLR4⁻/⁻ mice were stimulated with 100 nM RA for 30, 60 or 90 min. The cells were washed and homogenized in a lysis buffer containing 50 mM Tris (pH 8.0), 0.5% NP-40, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and a tablet of protease-inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Phosphorylated ERK1/2 (Thr202/Tyr204) or total ERK1/2, were detected with antibodies purchased from Cell Signaling Technology (Beverly, MA). After stripping of anti-phospho-ERK1/2 Abs using Western blot stripping buffer (Pierce, Rockford, IL) the membranes were reprobed with anti-ERK1/2 Abs. Densitometric analyses were performed by calculating the band intensity ratio of phosphorylated-ERK1/2 to total-ERK1/2 using the software ImageJ (National Institute of Health, Bethesda, MD).

**Competitive homing experiments**

Thy1.2⁺ CD8 T cells were activated with UT-DC or RA-DC. After 4 days, activated T cells were labeled with 5 μM CFSE or 10 μM CMTMR, as described. Briefly, cells were resuspended at 1x10⁷/ml, incubated with CFSE or CMTMR for 20 min at 37°C, and washed in an FBS gradient. 1-2 x 10⁷ cells from each population were mixed in a 1:1 ratio and injected into recipient C57Bl/6 Thy1.1⁺
mice. 18 h later the mice were euthanized, single cell suspensions were generated from spleen, MLN, intra-epithelial lymphocytes (IEL) or lamina propia (LP) from colon or small intestine (SI). Small bowel lamina propria cells were isolated after carefully dissecting out the Peyer’s patches. Cells samples were incubated with anti-Thy1.2 and analyzed on a FACScalibur (BD Biosciences) by gating on viable CD8^+Thy1.2^+ cells. The homing index (HI) was calculated as: HI=[T cells activated with RA-DC]_tissue/[T cells activated with UT-DC]_tissue: [T cells activated with RA-DC]_input/[T cells activated with UT-DC]_input.

**Statistical analysis**

Unless specified otherwise, data are presented as mean ± SEM and were analyzed using GraphPad Prism Software 5.0b. Statistics were calculated using either unpaired t test when comparing two groups or ANOVA with Dunnett’s or Bonferroni’s post-hoc test when comparing more than 2 groups. Significance was set at p < 0.05.

**References Supplemental Material**


