MyD88 and Retinoic Acid Signaling Pathways Interact to Modulate Gastrointestinal Activities of Dendritic Cells

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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. **METHODS:** 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.

Lymphocyte migration is a key event during intestinal inflammation.[1] Therefore, it is critical to understand how T and B cells migrate to the intestine and how their migration patterns could be manipulated for therapeutic purposes. The gut-homing receptors integrin $\alpha4\beta7$ and chemokine receptor CCR9 are required for T- and B-lymphocyte migration to the gut mucosa in the steady state and also during intestinal inflammation in mice and humans,[1,2] thus acting as molecular "ZIP codes" by controlling lymphocyte migration in a tissue-specific fashion.

DC from mesenteric lymph nodes (MLN-DC), Peyer's patches (PP-DC), and small intestine lamina propria (gut-associated DC), but not DC from extraintestinal sites, induce a high expression of gut-homing receptors on lymphocytes,[3–6] which is explained by their ability to metabolize vitamin A (retinol) into all-*trans* retinoic acid (RA). RA is necessary to imprint gut-homing lymphocytes,[5–7] to promote differentiation of IgA antibody-secreting cells (ASC)[5] and to control the balance between regulatory T cells and Th_{17} cells in the gut mucosa.[8,9] Therefore, given the essential role of RA in intestinal immune homeostasis, it is important to understand how the synthesis of RA is modulated in the gut mucosa.

It has been suggested that some nuclear receptor agonists can modulate RA synthesis in DC.[10] Here we show that RA controls RA-synthesizing capacity in DC in vitro and in vivo, inducing a positive feedback loop on its own synthesis and conferring DC with gut-specific imprinting properties. In addition, we found that RA-mediated DC education requires expression of the intracellular adaptor MyD88, which is conventionally associated with Toll-like receptor (TLR) and interleukin (IL)-1/IL-18 signaling,[11,12] suggesting a novel crosstalk between RA- and MyD88-dependent pathways.

Abbreviations used in this paper: ASC, antibody-secreting cells; DC, dendritic cells; ERK, extracellular signal–regulated kinase; GM-CSF, granulocyte-macrophage colony-stimulating factor; IEC, intestinal epithelial cells; IL, interleukin; LP-DC, lamina propria–derived dendritic cells; MAPK, mitogen-activated protein kinase; MLN-DC, mesenteric lymph node–derived dendritic cells; Mo-DC, monocyte-derived dendritic cells; mRNA, messenger RNA; PLN-DC, peripheral lymph node–derived dendritic cells; PPAR, peroxisome proliferator-activated receptor; PP-DC, Peyer patches–derived dendritic cells; RA, *all-trans* retinoic acid; RA-DC, dendritic cells pretreated with all-*trans* retinoic acid; RALDH, retinal dehydrogenase; RAR, retinoic acid receptor; RXR, retinoid X receptor; TLR, Toll-like receptor; UT-DC, untreated spleen dendritic cells; VAD, vitamin A–deficient.

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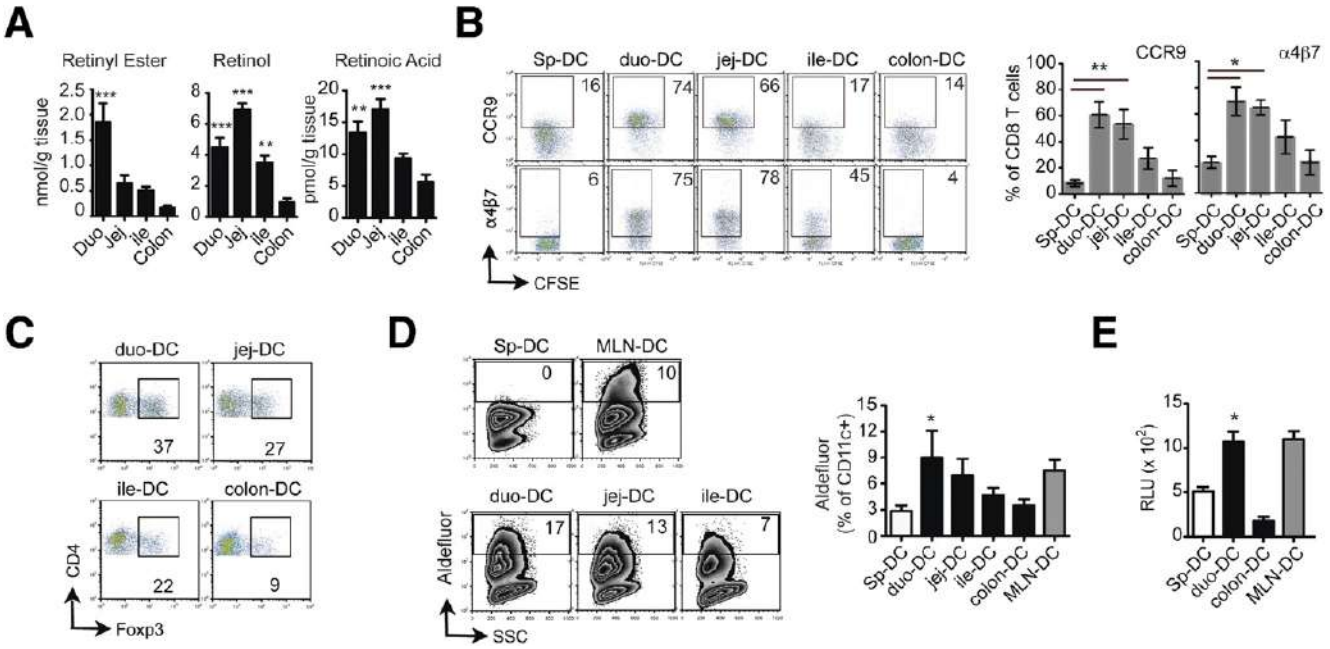

Figure 1. DC ability to imprint gut homing correlates with retinoid levels in the gut. (*A*) Quantification of retinyl esters, retinol, and RA in duodenum (duo), jejunum (jej), ileum (ile), and colon (n = 6). (*B*) LP-DC or PP-DC from duo, jej, ile, and colon were used to activate naïve CD8 T cells. After 4−5 days, T cells were analyzed for $\alpha 4\beta 7$ and CCR9 expression (n = 5). (*C*) DC were used to activate naïve CD4 T cells. After 4 days, T cells were analyzed for Foxp3 expression. Fluorescence-activated cell sorting (FACS) plots are representative of 2 independent experiments. (*D*) Retinal dehydrogenase (RALDH) activity in DC (n = 4). (*E*) Luciferase activity in DC from DR5-luciferase mice (n = 4). Mean ± SEM; $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

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 1*A*). 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 ($\alpha 4\beta 7$ and CCR9) and Foxp3^{+} T cells as compared to their counterparts in ileum, colon, or spleen (Figure 1*B* 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 1*B*), 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 1*D*), which was not explained by different proportions of CD103^{+} DC (Supplementary Figure 1*C*). Of note, expression of *Aldh1a2* messenger RNA (mRNA) (encoding RALDH2) was not significantly different among DC from different intestinal segments and their relative mRNA levels were lower than those found in MLN-DC (Supplementary Figure 1*D*), 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 1*E*). However, consistent with their exposure to high levels of RA, DC from the proximal small intestine exhibited higher luciferase activity than distal DC (Figure 1*E*).

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

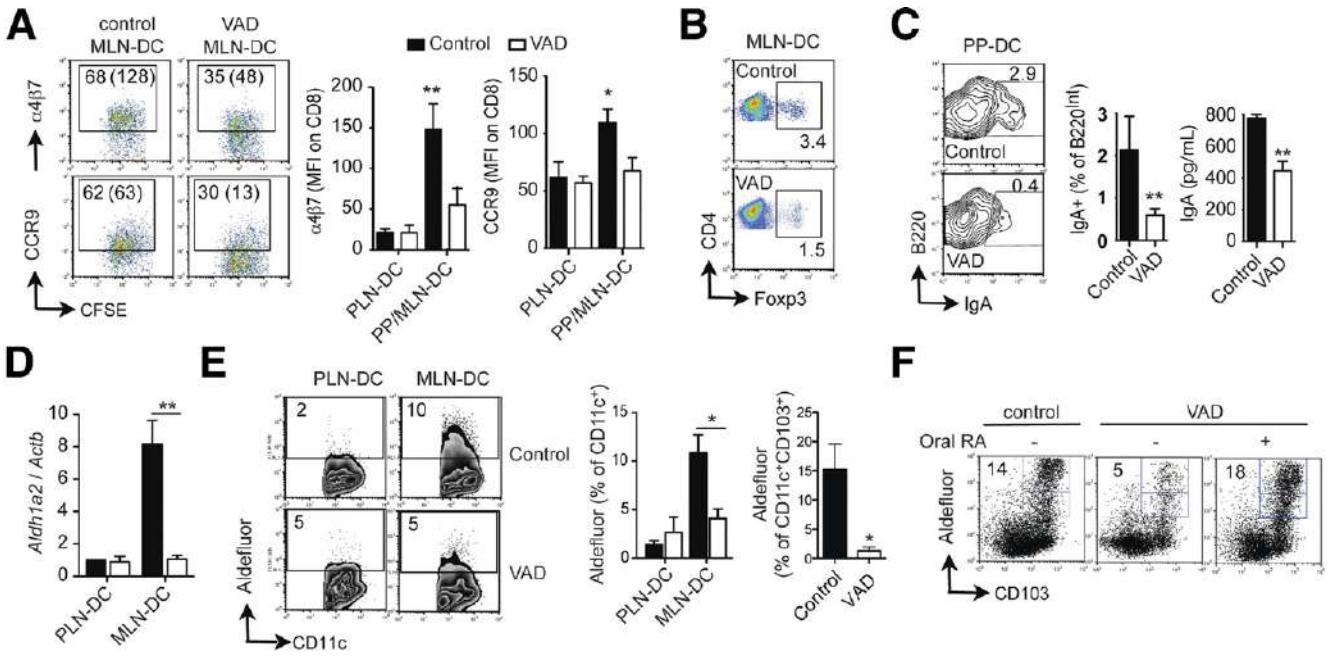

Figure 2. RA is necessary in vivo for gut-associated DC education. DC were isolated from mice on a VAD or control diet. (*A*) DC were used to activate naïve CD8 T cells. After 4−5 days, T cells were analyzed for $\alpha4\beta7$ and CCR9 expression (n = 4). (*B*) DC were used to activate naïve CD4 T cells. After 4−5 days, T cells were analyzed for Foxp3 expression. FACS plots are representative of 2 independent experiments. (*C*) DC were cocultured with naïve B cells activated with anti-IgM plus IL-5. After 4 days, cocultures were analyzed for intracellular IgA in $B220^{Int}$ cells and IgA in the supernatant (n = 4). (*D*) *Aldh1a2* mRNA and (*E*) RALDH activity in $CD11c^+$ and $CD11c^+CD103^+$ DC (n = 5). (*F*) RALDH activity in MLN $CD11c^+$ cells from control or VAD mice ± oral RA supplementation. FACS plots are representative of 2 independent experiments. Mean ± SEM; $^*P < .05$; $^{**}P < .01$.

on T cells as compared to their counterparts from mice on a vitamin A−sufficient diet (Figure 2*A*). In addition, MLN-DC and PP-DC from VAD mice were impaired in inducing $Foxp3^+$ T cells (Figure 2*B*) and IgA-ASC (Figure 2*C*), respectively. Moreover, MLN-DC from VAD mice showed a marked reduction in *Aldh1a2* mRNA expression (Figure 2*D*) and an impaired RALDH activity in total and $CD103^+$ MLN-DC (Figure 2*E*), which was rescued by oral RA supplementation (Figure 2*F*). 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 3*A* and Supplementary Figure 2*A* 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-(1′H-**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 2*C*), as well as *ALDH1A2* mRNA in human monocyte-derived DC (Mo-DC) (Supplementary Figure 2*D*), 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 3*B*), which was also observed when treating PLN-DC and bone marrow−DC with RA (Supplementary Figure 2*E*), indicating that RA-DC acquired RA-synthesizing capacity. Importantly, RA-DC induced significantly higher levels of gut-homing receptors $\alpha4\beta7$ and CCR9 on activated T cells as compared to UT-DC, which was abrogated when T cells were activated in retinol-free media (Figure 3*C*) or in the presence of the RALDH inhibitor diethylaminobenzaldehyde (DEAB)[17] (Figure 3*D*), 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 2*F*). Adding the RAR-inhibitor LE540 during the coculture abrogated gut-homing imprinting by RA-DC (Supplementary Figure 2*G*), 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-

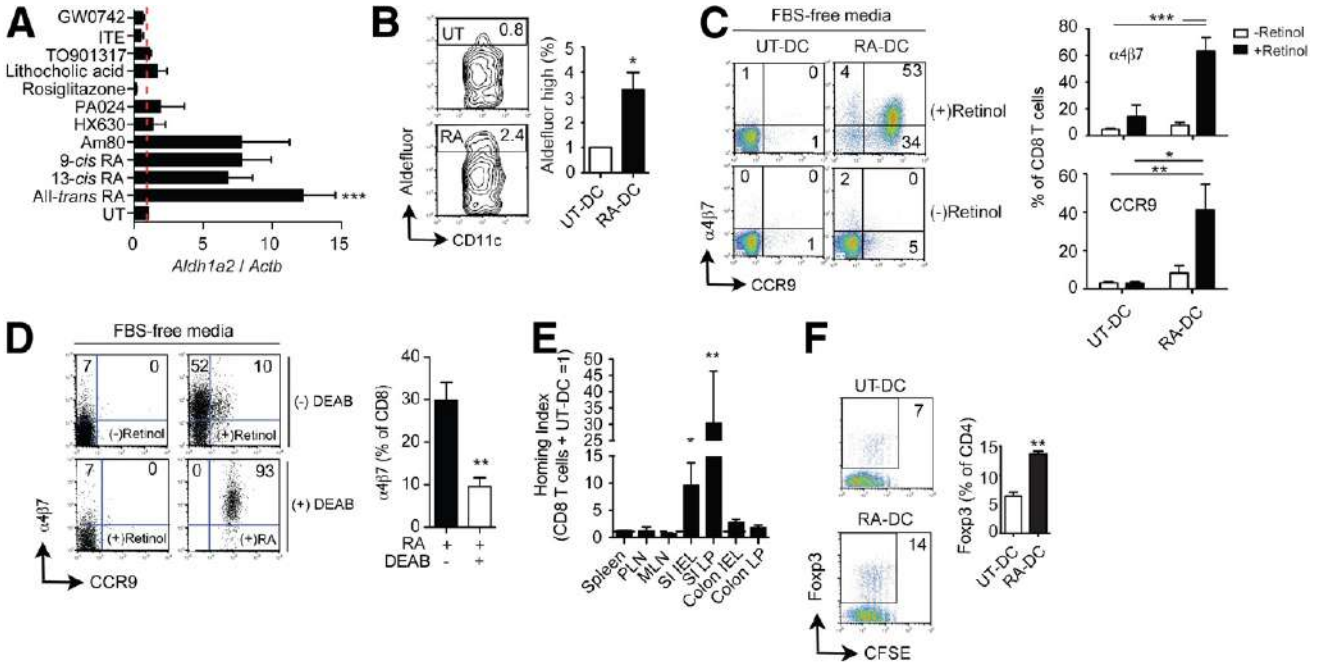

Figure 3. RA is sufficient to confer DC with RA synthesizing capacity in vitro. (*A*) *Aldh1a2* mRNA in spleen-DC incubated for 24 hours with 100 nM all-*trans* RA or 100 nM of the indicated nuclear receptor agonists. HX630 and PA024 were used at 1 or 10 μM, with similar results (n = 5−10). (*B*−*F*) spleen-DC were incubated for 24 hours with or without 100 nM RA (RA-DC and UT-DC, respectively) and analyzed for (*B*) RALDH activity (n = 5) or (*C*) washed, pulsed with antigen, and used to activate naïve CD8 T cells from T-cell receptor transgenic OT-1xRAG2$^{-/-}$ mice in fetal bovine serum−free media ± 50 nM retinol. After 4−5 days, T cells were analyzed for $\alpha4\beta7$ and CCR9 expression (n = 7). (*D*) Cocultures were performed as described in (*C*) either in the presence or absence of the RALDH inhibitor diethylaminobenzaldehyde (DEAB) (n = 7). (*E*) Competitive homing experiment between CD8 T cells activated with RA-DC or UT-DC (n = 5). (*F*) Naïve CD4 T cells from OT-2xRAG2$^{-/-}$ mice were activated with UT-DC or RA-DC. Five days later CD4 T cells were analyzed for Foxp3 expression (n = 3).

pared to those activated with UT-DC (Figure 3*E*). In addition, analogous to gut-associated DC[8,9] RA-DC also promoted the induction of Foxp3$^+$ regulatory T cells (Figure 3*F*).

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.[19] 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 3*A* 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 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 3*D*). 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 3*E*). Moreover, MLN-DC maintained the same high RALDH activity regardless of whether the mice were supplemented with oral RA (Supplementary Figure 3*F*). 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 4*A*). As expected, PLN-DC from control mice showed much lower levels of *Aldh1a2* mRNA and RALDH activity as compared to MLN-DC (Figure 4*B* and *C*, Supplementary Figure 4*A*). By contrast, PLN-DC from mice treated with oral RA exhibited significantly higher levels of *Aldh1a2* mRNA and RALDH activity than PLN-DC from control mice. In addition, PLN-DC from RA-treated mice were able to induce gut-homing receptors on activated T cells in vitro, an effect that was dependent

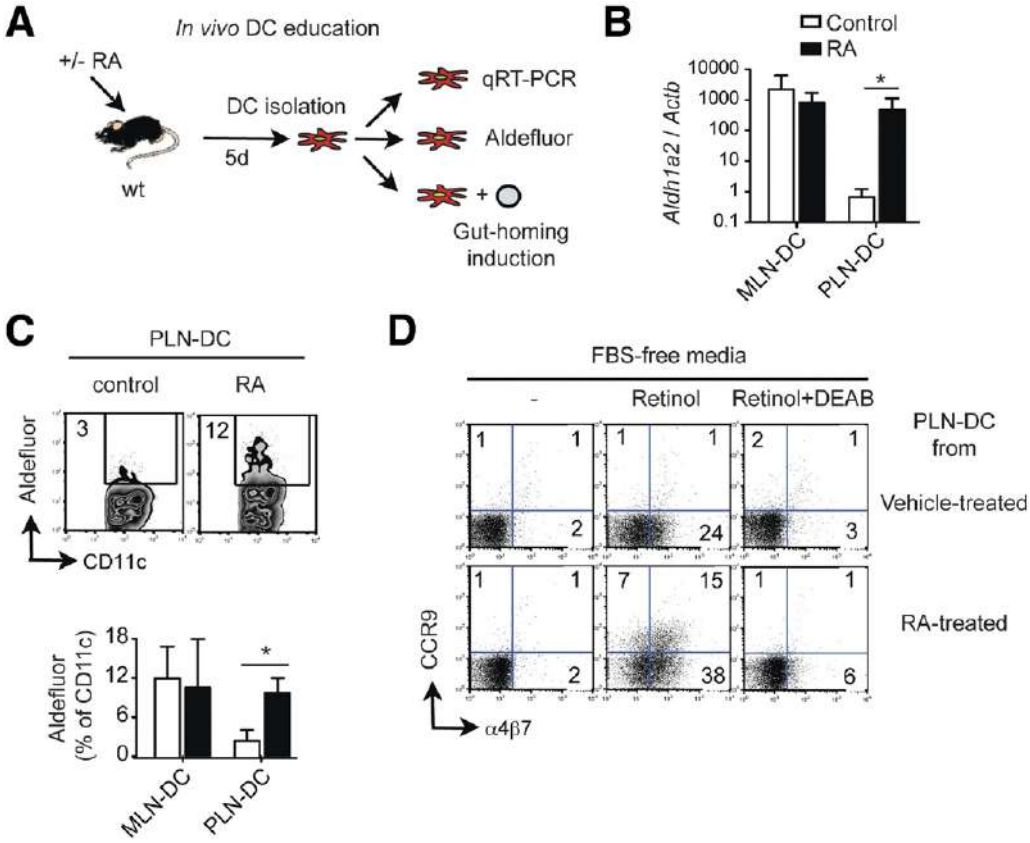

Figure 4. RA is sufficient to confer DC with RA synthesizing capacity in vivo. (*A*) Wild-type mice were supplemented orally with RA (400 μg/dose) every day for 5 days. After that, MLN-DC and PLN-DC were isolated and analyzed for the expression of (*B*) *Aldh1a2* mRNA and (*C*) RALDH activity (n = 5). (*D*) PLN-DC from control or RA-treated mice were used to activate naïve CD8 T cells in fetal bovine serum−free media ± retinol and either in the presence or absence of DEAB. FACS plots are representative of 2 independent experiments. Mean ± SEM; $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

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

We could not detect RA in PLN from untreated animals, even using the most sensitive methods,[16] whereas RA was readily detected in PLN from RA-treated animals with levels approaching those found in MLN from untreated animals (Supplementary Figure 4*B*). 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 4*C*). 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 (Supplementary Figure 4*D*). Thus, oral RA treatment increases RA concentration and RA-dependent activity in PLN to levels comparable to those in the normal MLN environment, suggesting that RA or other RAR agonists, such as 13-*cis* RA (isotretinoin) (Figure 3*A*), could be used as "mucosal adjuvants" to increase gut-associated immune responses in peripheral compartments.

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 5*A*). 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 5*B*), 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 5*C*), 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

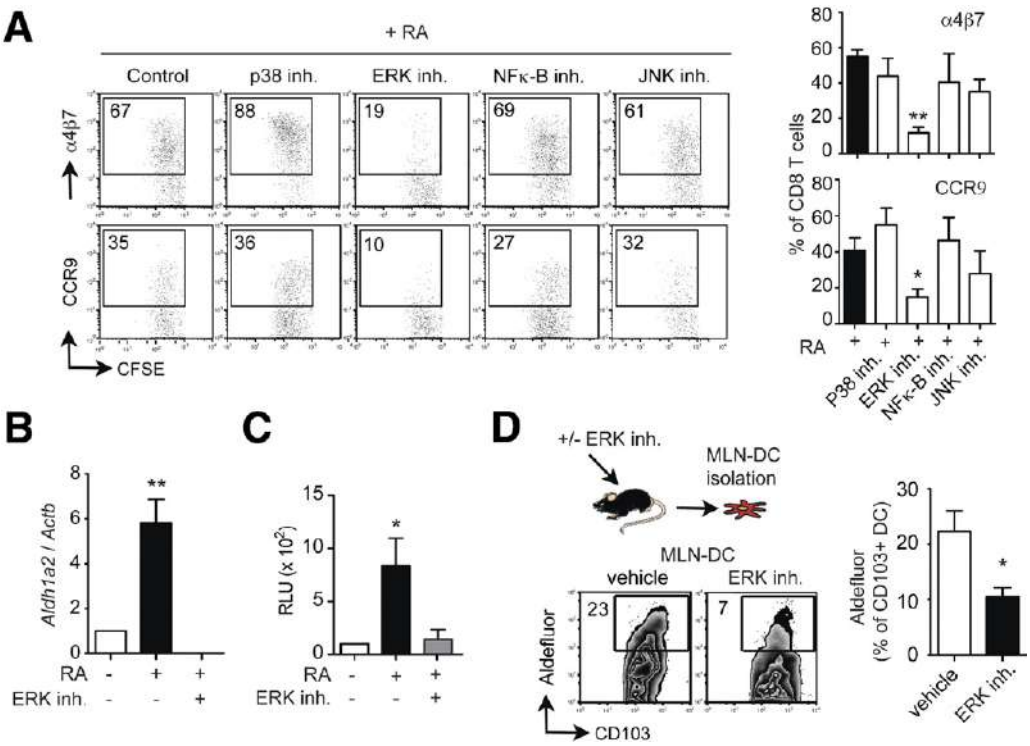

Figure 5. DC education requires ERK/MAPK signaling. (*A*–*C*) Spleen-DC were incubated for 24 hours with or without 100 nM RA and in the presence or the absence of inhibitors for p38 (SB203580, 10 μM), ERK (U0126, 10 μM), nuclear factor–κB (SN50, 50 μM), or c-Jun-N-terminal kinase (SP600125, 50 μM). (*A*) DC were used to activate naïve CD8 T cells. After 4–5 days, T cells were analyzed for α4β7 and CCR9 expression (n = 9). (*B*) *Aldh1a2* mRNA expression in DC (n = 3). (*C*) Spleen-DC from DR5-luciferase mice were incubated for 24 hours with or without RA and in the presence or absence of the ERK inhibitor (U0126, 10 μM) and analyzed for their luciferase activity (n = 3). (*D*) Wild-type mice were orally treated with the ERK inhibitor PD0325901 (25 μg/g/dose) every other day for 6 days. After that, CD11c$^+$ MLN-DC were analyzed for CD103 expression and RALDH activity (n = 5). Mean ± SEM; $^*P < .05$; $^{**}P < .01$.

been used in vivo.[15] 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 5*D*), 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[20] and that RA-mediated DC education also requires ERK signaling, we explored whether TLR signals might modulate RA effects on DC.[11] Consistent with this possibility, spleen-DC from mice lacking MyD88 (essential for most TLR signaling[11]) 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 6*A*), and the induction of *Aldh1a2* mRNA was completely abrogated in MyD88$^{-/-}$ RA-DC (Figure 6*B*). Similarly, *Tgm2* mRNA (encoding tissue transglutaminase, a well-known RA target gene[21]) was not induced in MyD88$^{-/-}$ RA-DC, whereas it was readily induced in wild-type RA-DC (Figure 6*B*), 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 6*C* and Supplementary Figure 5*A*). 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 5*B*). 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,[10] was expressed at similar levels in MyD88$^{-/-}$ and wild-type PP-DC (Supplementary Figure 5*C*). 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[22] (Supplementary Figure 5*D*).

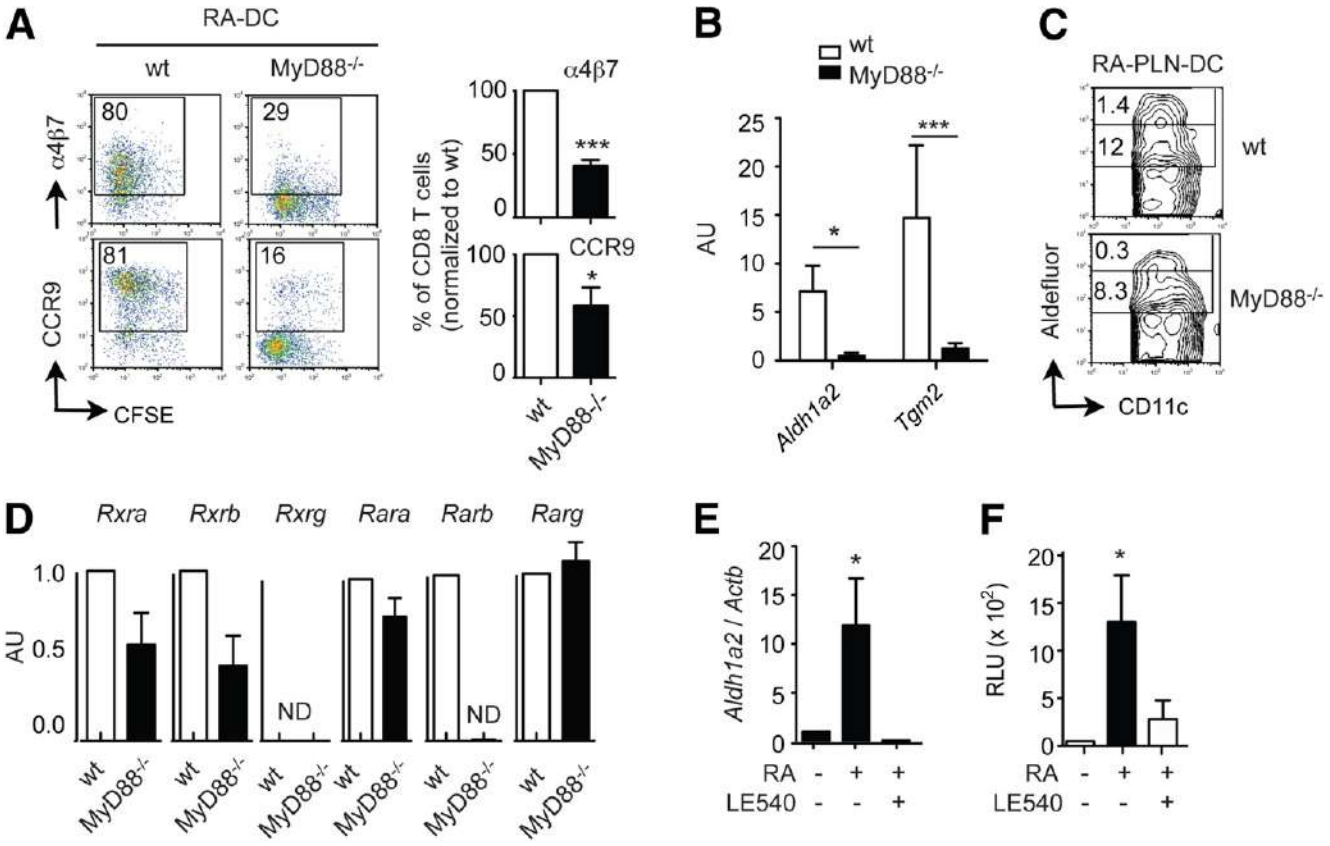

Figure 6. MyD88 is required for RA-mediated DC education. (*A, B*) Wild-type or MyD88$^{-/-}$ spleen-DC were incubated with 100 nM RA for 24 hours. (*A*) DC were used to activate naïve CD8 T cells. After 4−5 days, T cells were analyzed for $\alpha4\beta7$ and CCR9 expression (n = 5). (*B*) *Aldh1a2* (n = 4) or *Tgm2* (n = 5) mRNA expression in DC. (*C*) Wild-type or MyD88$^{-/-}$ mice were supplemented orally with RA (400 μg/dose) every day for 5 days and PLN-DC were analyzed for RALDH activity (n = 7). (*D*) Spleen-DC from wild-type or MyD88$^{-/-}$ mice were analyzed for their expression of *Rxra, Rxrb. Rxrg, Rara, Rarb,* and *Rarg* mRNA (n = 4−6). ND, not detected. (*E*) Spleen-DC were incubated for 24 hours with or without 100 nM RA and either in the presence or absence of the RARβ inhibitor LE540 (1 μM) and then analyzed for *Aldh1a2* mRNA expression (n = 3). (*F*) Spleen-DC from DR5-luciferase mice were incubated for 24 hours with or without 100 nM RA and either in the presence or absence of LE540 and then analyzed for their luciferase activity (n = 3). Mean ± SEM; $^{*}P < .05$; $^{***}P < .001$.

Interestingly, RA induced *Tlr1* and *Tlr2* mRNA (Supplementary Figure 5*E*). 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 3*C* and *D*, Supplementary Figure 5*E*), 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 5*F* 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 6*D*), 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 6*E*) and RA-induced luciferase activity in spleen-DC from DR5-luciferase mice (Figure 6*F*). 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

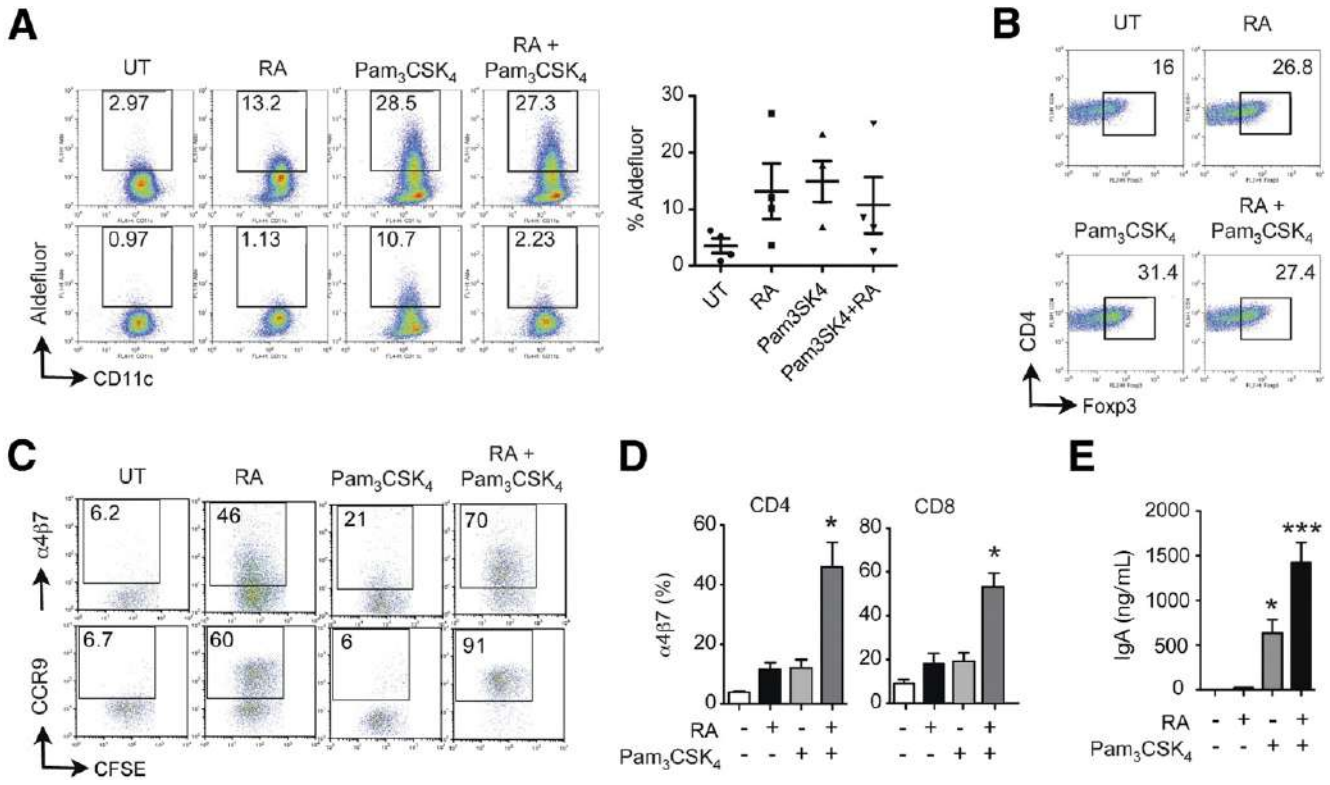

Figure 7. TLR signals contribute to RA-mediated human and murine DC education. (*A*) Mo-DC from human healthy donors were treated with either RA (100 nM), Pam_3CSK_4 (0.5 μg/mL), or both from day 3 of differentiation. RALDH activity was analyzed at day 6 in $CD11c^+$ cells (n = 4). FACS plots show representative results in Mo-DC from 2 donors. (*B*) Mo-DC pretreated with either RA, Pam_3CSK_4, or both, were washed and cultured with allogenic CD4 T cells activated with anti-CD3 plus anti-CD28 and transforming growth factor−β1 (TGF-β1; 2 ng/mL). After 4 days, CD4 T cells were analyzed for Foxp3 expression. FACS plots show 1 representative experiment out of 2. (*C*) Spleen-DC were incubated for 24 hours in the presence of 100 nM RA, Pam_3CSK_4 (0.5 μg/mL), or both, washed and used to activate naive CD8 T cells. After 4 days, T cells were analyzed for their expression of $\alpha4\beta7$ and CCR9. FACS plots are representative of 3 experiments. (*D*) Human Mo-DC were treated at day 6 with or without 100 nM of RA, Pam_3CSK_4 (0.5 μg/mL), or both, for 24 hours, washed and cocultured 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 $\alpha4\beta7$ (n = 3). (*E*) Spleen-DC were incubated for 24 hours in the presence of 100 nM RA, Pam_3CSK_4 (0.5 μg/mL), or both, washed and cocultured with naïve B cells activated with anti-IgM plus IL-5. After 4 days, IgA levels were measured in the culture supernatants (n = 7). Mean ± SEM; $^*P < .05$; $^{***}P < .001$.

TLR1/2-agonist (Pam_3CSK_4) exhibited higher RALDH activity as compared to untreated Mo-DC (Figure 7*A*). The increased RALDH activity in Mo-DC correlated with their ability to induce Foxp3 in human CD4 T cells (Figure 7*B*). Moreover, mouse spleen-DC or human Mo-DC treated with RA plus Pam_3CSK_4 induced higher proportions of $\alpha4\beta7^+$ and $CCR9^+$ T cells as compared to DC treated with either RA or Pam_3CSK_4 alone (Figure 7*C* 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 7*E*), 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′-monooxygenase 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,[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[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 RALDH1[30,31] and can produce RA.[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,[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.[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$^{-/-}$ 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 β-c subunit, which is shared by GM-CSF, IL-3, and IL-5 receptors.[17]

Similar to TLR2 stimulation,[20] 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.[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-specific 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 specialization integrates signals derived from dietary components acting on nuclear receptors and the intestinal microbiota acting via pathogen-recognition receptors. These signals could eventually be manipulated to improve vaccination strategies aimed at enhancing intestinal immunity.

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

1. Mora JR. Homing imprinting and immunomodulation in the gut: role of dendritic cells and retinoids. Inflamm Bowel Dis 2008;14:275–289.
2. Eksteen B, Adams DH. GSK-1605786, a selective small-molecule antagonist of the CCR9 chemokine receptor for the treatment of Crohn's disease. IDrugs 2010;13:472–781.
3. Mora JR, Bono MR, Manjunath N, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. Nature 2003;424:88–93.
4. Johansson-Lindbom B, Svensson M, Wurbel MA, et al. Selective generation of gut tropic T cells in gut-associated lymphoid tissue (GALT): requirement for GALT dendritic cells and adjuvant. J Exp Med 2003;198:963–969.
5. Mora JR, Iwata M, Eksteen B, et al. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science 2006;314:1157–1160.
6. Eksteen B, Mora JR, Haughton EL, et al. Gut homing receptors on CD8 T cells are retinoic acid dependent and not maintained by liver dendritic or stellate cells. Gastroenterology 2009;137:320–329.
7. Iwata M, Hirakiyama A, Eshima Y, et al. Retinoic acid imprints gut-homing specificity on T cells. Immunity 2004;21:527–538.
8. Mucida D, Park Y, Kim G, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science 2007;317:256–260.
9. Sun CM, Hall JA, Blank RB, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J Exp Med 2007;204:1775–1785.
10. Szatmari I, Pap A, Ruhl R, et al. PPARgamma controls CD1d expression by turning on retinoic acid synthesis in developing human dendritic cells. J Exp Med 2006;203:2351–2362.
11. Kawai T, Akira S. TLR signaling. Semin Immunol 2007;19:24–32.
12. Adachi O, Kawai T, Takeda K, et al. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity 1998;9:143–150.
13. Svensson M, Johansson-Lindbom B, Zapata F, et al. Retinoic acid receptor signaling levels and antigen dose regulate gut homing receptor expression on CD8+ T cells. Mucosal Immunol 2008;1:38–48.
14. O'Byrne SM, Wongsiriroj N, Libien J, et al. Retinoid absorption and storage is impaired in mice lacking lecithin:retinol acyltransferase (LRAT). J Biol Chem 2005;280:35647–35657.
15. Lee SH, Hu LL, Gonzalez-Navajas J, et al. ERK activation drives intestinal tumorigenesis in Apc(min/+) mice. Nat Med 2010;16:665–670.
16. Kane MA, Napoli JL. Quantification of endogenous retinoids. Methods Mol Biol 2010;652:1–54.
17. Yokota A, Takeuchi H, Maeda N, et al. GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity. Int Immunol 2009;21:361–377.
18. Saurer L, McCullough KC, Summerfield A. In vitro induction of mucosa-type dendritic cells by all-trans retinoic acid. J Immunol 2007;179:3504–3514.

19. Sun D, Ding A. MyD88-mediated stabilization of interferon-gamma-induced cytokine and chemokine mRNA. Nat Immunol 2006;7:375–381.
20. Manicassamy S, Ravindran R, Deng J, et al. Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity. Nat Med 2009;15:401–409.
21. Moore WT Jr, Murtaugh MP, Davies PJ. Retinoic acid-induced expression of tissue transglutaminase in mouse peritoneal macrophages. J Biol Chem 1984;259:12794–12802.
22. Schug TT, Berry DC, Shaw NS, et al. Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. Cell 2007;129:723–733.
23. Canon E, Cosgaya JM, Scsucova S, et al. Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. Mol Biol Cell 2004;15:5583–5592.
24. Li Y, Hashimoto Y, Agadir A, et al. Identification of a novel class of retinoic acid receptor beta-selective retinoid antagonists and their inhibitory effects on AP-1 activity and retinoic acid-induced apoptosis in human breast cancer cells. J Biol Chem 1999;274:15360–45366.
25. Tezuka H, Abe Y, Iwata M, et al. Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. Nature 2007;448:929–933.
26. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and D take centre stage. Nat Rev Immunol 2008;8:685–698.
27. von Lintig J. Colors with functions: elucidating the biochemical and molecular basis of carotenoid metabolism. Annu Rev Nutr 2010;30:35–56.
28. Herr FM, Wardlaw SA, Kakkad B, et al. Intestinal vitamin A metabolism: coordinate distribution of enzymes and CRBP(II). J Lipid Res 1993;34:1545–1554.
29. Stenstad H, Svensson M, Cucak H, et al. Differential homing mechanisms regulate regionalized effector CD8alphabeta+ T cell accumulation within the small intestine. Proc Natl Acad Sci U S A 2007;104:10122–10127.
30. Lampen A, Meyer S, Arnhold T, et al. Metabolism of vitamin A and its active metabolite all-trans-retinoic acid in small intestinal enterocytes. J Pharmacol Exp Ther 2000;295:979–985.
31. Iliev ID, Mileti E, Matteoli G, et al. Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. Mucosal Immunol 2009;2:340–350.
32. Frota-Ruchon A, Marcinkiewicz M, Bhat PV. Localization of retinal dehydrogenase type 1 in the stomach and intestine. Cell Tissue Res 2000;302:397–400.
33. Bhat PV. Retinal dehydrogenase gene expression in stomach and small intestine of rats during postnatal development and in vitamin A deficiency. FEBS Lett 1998;426:260–262.
34. Battle TE, Roberson MS, Zhang T, et al. Retinoic acid-induced blr1 expression requires RARalpha, RXR, and MAPK activation and uses ERK2 but not JNK/SAPK to accelerate cell differentiation. Eur J Cell Biol 2001;80:59–67.

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Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Figure 1

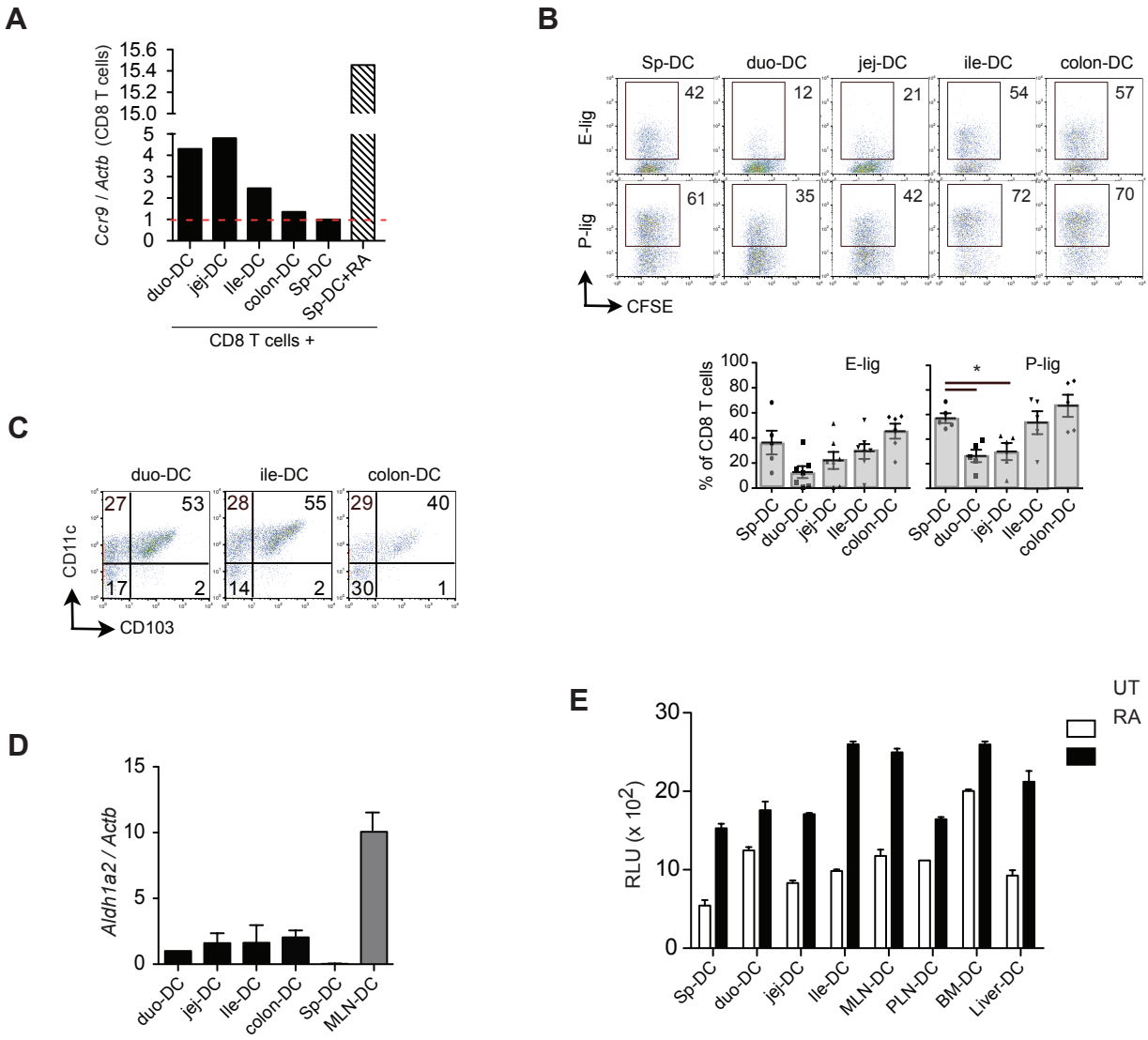

Fig. S1. (**A-B**) CD8 T cell were activated with Sp-DC, PP-DC from duodenum (duo), jejunum (jej), ileum (ile) or colon LP-DC and analyzed after 4 days. (**A**) Expression of *Ccr9* mRNA in activated T cells. As positive control, 10 nM RA was supplemented to some co-cultures with Sp-DC. Graph is representative 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

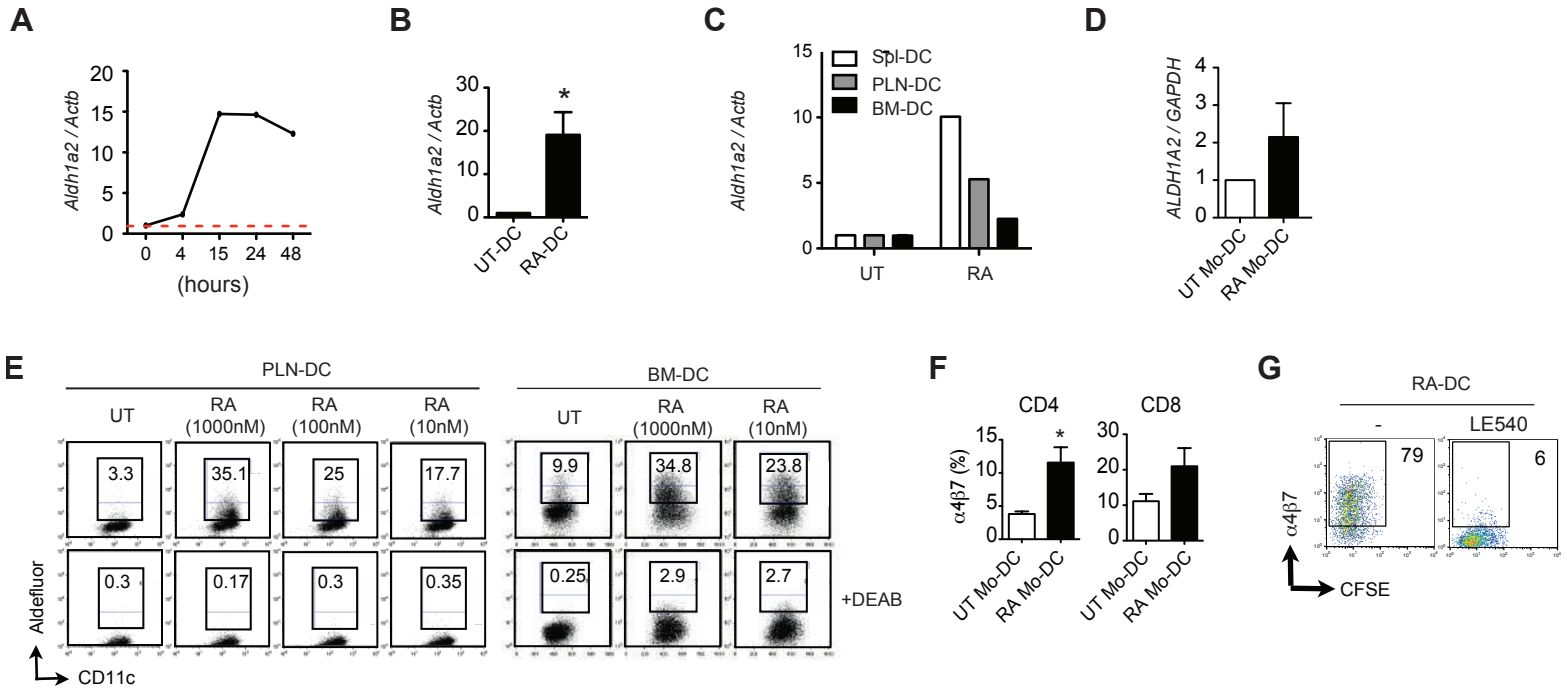

Fig. S2. (**A-B**) Sp-DC were treated with (RA-DC) or without (UT-DC) 100 nM RA. (A) Kinetics for *Aldh1a2* mRNA expression and (**B**) pooled data at 24 h (n=7). (**C**) Aldh1a2 mRNA levesl on Sp-DC, PLN-DC or BM-DC treated or not with RA (1000 nM). (**D**) Human monocytes were differentiated to DC (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 diferentiated Bone marroe-derived DC (BM-DC) were treated or not with different concentration of RA. Upon 24 h, RALDH activity was measure 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.

Supplementary Figure 3

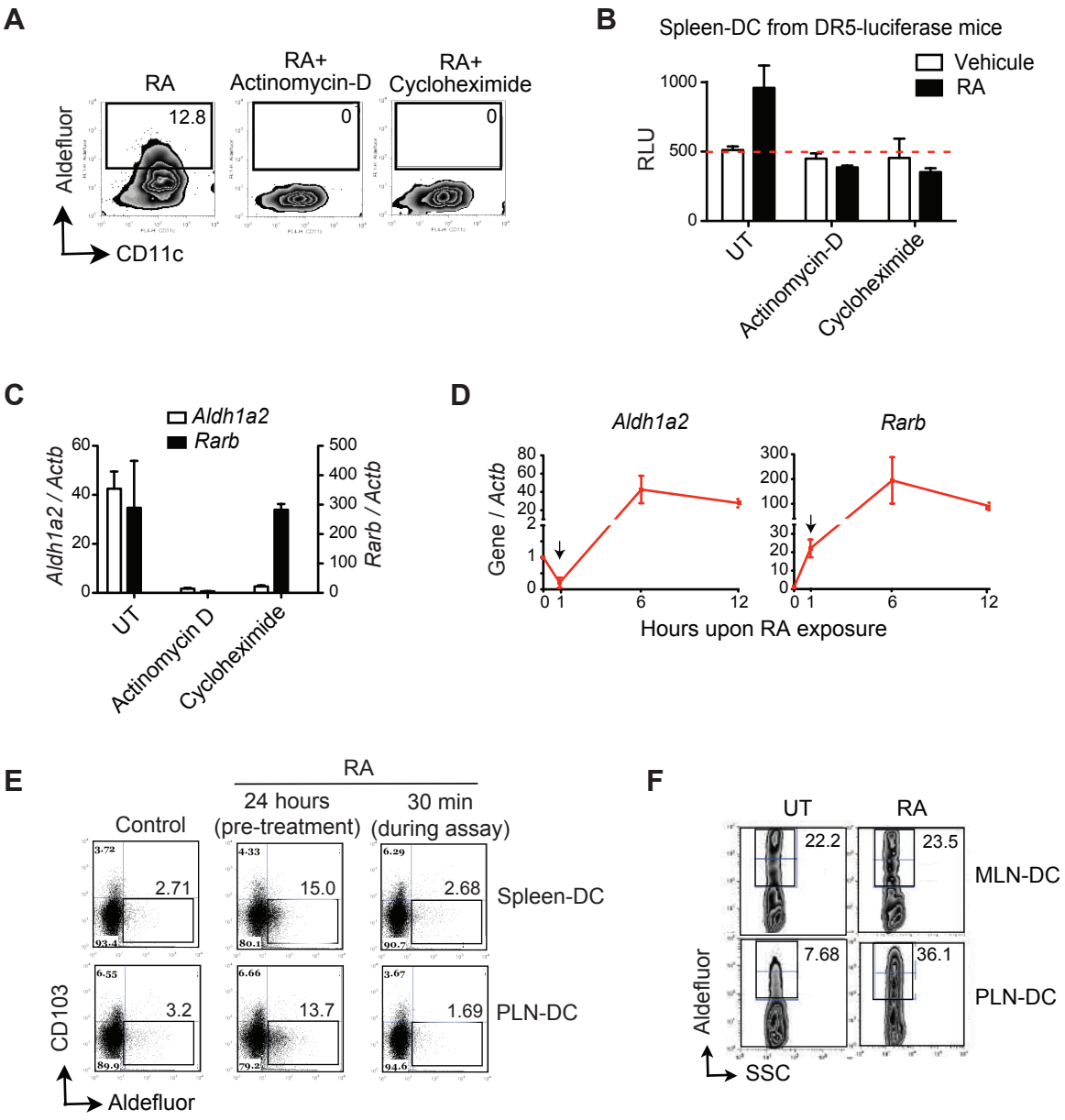

Fig. S3. (**A-D**) Spleen-DC from DR5-luciferase mice were treated or not with 100 nM RA and in the presence or absence of the transcription inhibitor actinomycin-D (1 μg/ml) or the translation inhibitor cycloheximide (10 μg/ml). After 20 hours the cells were assessed for their Raldh activity (Aldefluor 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).

Supplementary Figure 4

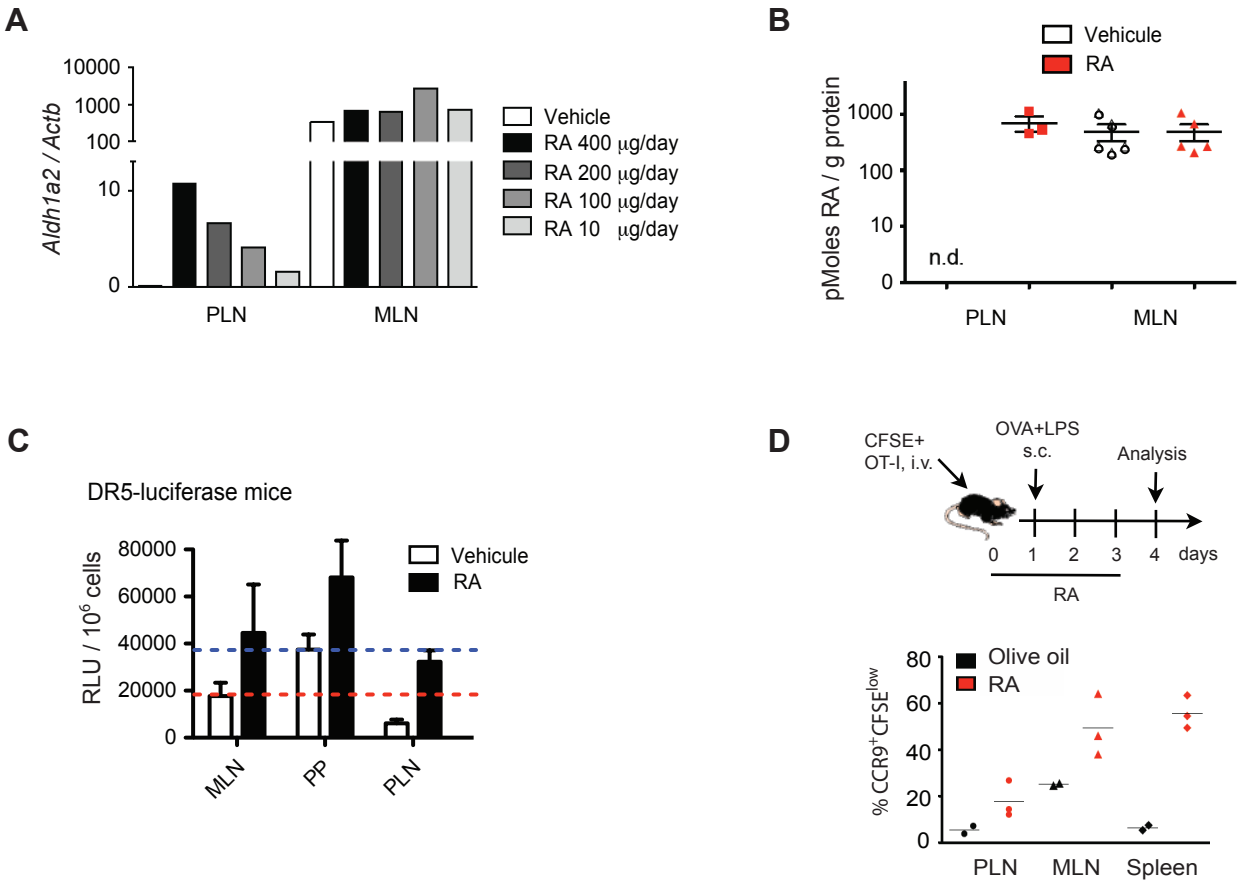

Fig. S4. (**A**) Wild type mice were orally treated with different concentration of RA (10-400 µg/day) for 4 days. PLN-DC and MLN-DCwere isolated at day 4 and analyzed for their expression of *Aldh1a2* mRNA. (**B-C**) Wild type mice (**B**) or DR5-luciferase mice (**B**) were treated with 400 µg/day 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 vehicule (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

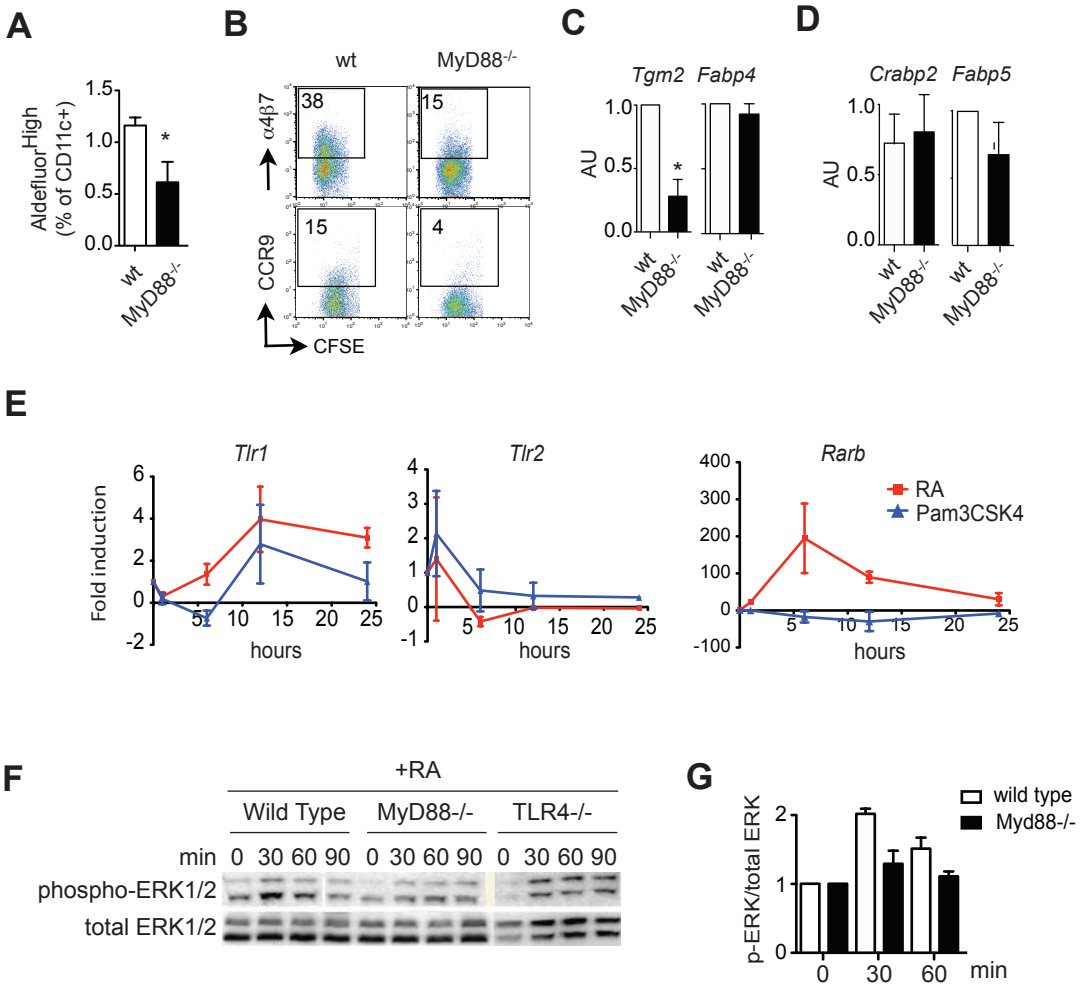

Fig. S5. (**A**) Wild type or MyD88$^{-/-}$ mice were supplemented orally with RA (400 µg/dose) every other day for 6 days. After that, PLN-DC were analyzed for Raldh activity (n=7). (**B**) Sp-DC from RA-supplemented wild type or MyD88$^{-/-}$ mice were used to activate naïve CD8 T cells. After 4 days 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

Supplementary Figure 6

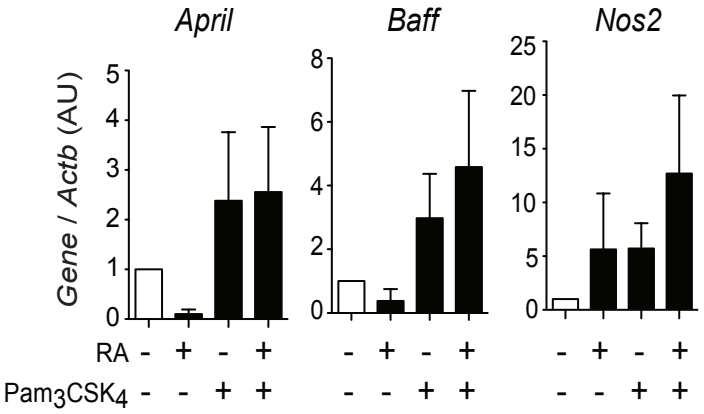

Fig. S6. Sp-DC were incubated for 24 h with or without 100 nM of RA, Pam_3CSK_4 (0.5 μg/ml) or both and then analyzed for their expression of *April*, *Baff* and *Nos2* mRNA (n=3).

Supplementary Figure 7

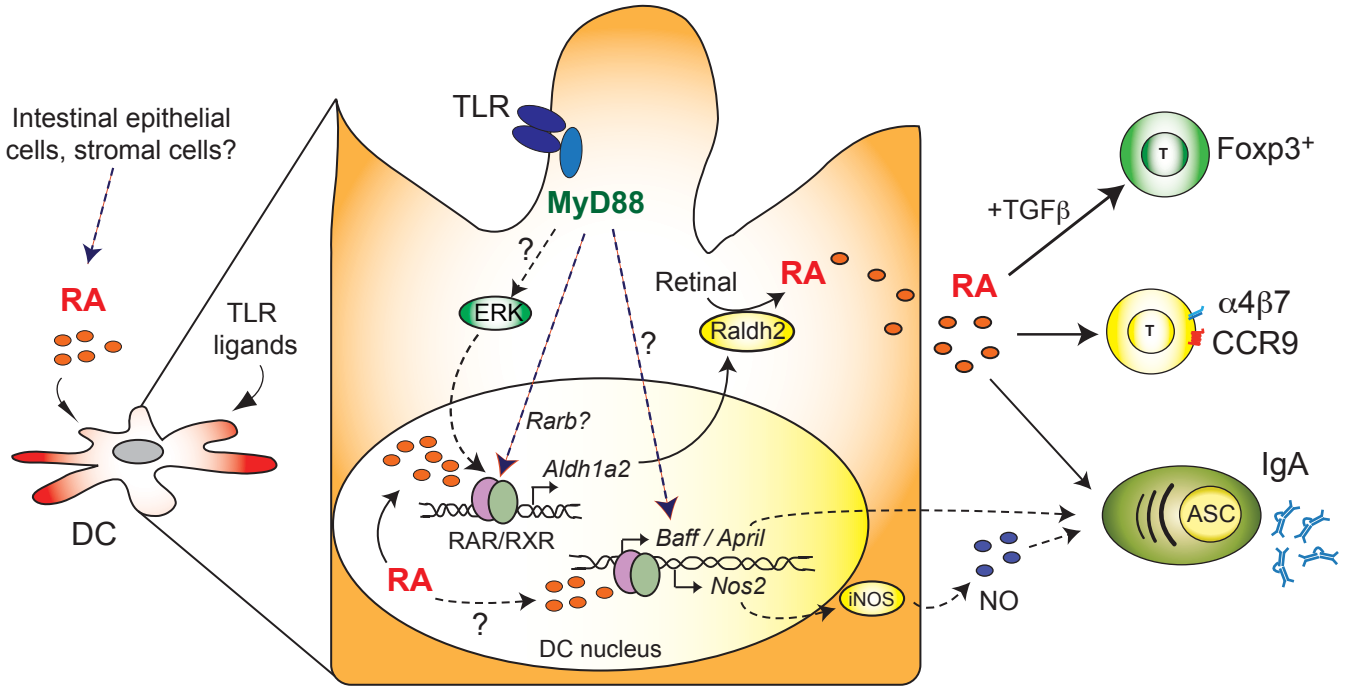

Fig. S7. Model for RA-mediated DC education. RA acts on DC via RAR-RXR nuclear receptors to induce *Aldh1a2* expression (encoding Radh2). Raldh2 metabolizes retinal into RA, which induces gut-homing receptors on T cells. In addition, RA potentiates the differentiation of IgA-ASC and Foxp3$^+$ T_{REG}. 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 RARβ) 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} *in vitro*, an effect that was partially dependent on RA and TGF-β [3]. However, since there are no studies assessing the specific contribution of IEC-derived RA in gut-associated DC education *in vivo*, the physiological relevance of IEC in this context remains to be determined.

Although our data show that RA readily upregulates *Aldh1a2* mRNA (encoding Raldh2), RA did not consistently induce *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$\alpha^{-/-}$, 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); PPARβ/δ-agonist GW0742 (Tocris, Ellisville, MI); AHR-agonist 2-(19H-indole-39-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) (Tocris, Ellisville, MI); PPARγ-agonist Rosiglitazone (Cayman, Ann Arbor, MI); RXR-agonists PA024 and HX630; and RARα/β-agonist Am80 were provided by Dr. Hiroyuki Kagechika (Tokyo Medical and Dental University, Japan); Raldh inhibitor 4-(diethylamino)-benzaldehyde (DEAB) (Stemcell, Vancouver); RARβ-antagonist LE540 (Wako Chemicals USA, Richmond, VA) was dissolved in DMSO or ethanol (1 mM stocks) and used at 1 μM final concentration. The following mAb used to label murine cells were purchased from BD Biosciences (San Jose, CA): anti-α4b7 (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 (Pam_3CSK_4) 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 [19]. The ERK1/2-inhibitor PD0325901 used for *in vivo* experiments [20] 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). $LCMVgp_{33–41}$ 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 [12, 21]. 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 MAbs 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 P14xTCR$\alpha^{-/-}$ or OT-1xRAG2$^{-/-}$ mice, DC were pulsed for 2 h with 100 nM $LCMVgp_{33–41}$ 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 [21]. 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. $1x10^5$ 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 10^7 cells/ml in DMEM + 1% FBS + 20 mM Hepes, incubated with 2.5 mM CFSE for 20 min at 37ºC 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) [12]. $1x10^5$ 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 $B220^{Int}$ B cells/ plasmablasts were analyzed by flow cytometry for intracellular IgA expression and for IgA in the culture supernatant [12].

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) [20]. 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 (5-6x10^7) 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 [12].

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* (Mm00436410_m1), *Tgm2* (Mm00436980_m1), *Fabp5* (Mm00783731_s1), *CrabpII* (Mm00801693_g1), *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 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 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 (Thry202/Tyr204) or total ERK1/2, were detected with antibodies purchased from Cell SignalingTechnology (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 [12, 21]. Briefly, cells were resuspended at 1x10^{7}/ml, incubated with CFSE or CMTMR for 20 min at 37ºC, and washed in an FBS gradient. 1-2 x 10^{7} 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

1. Stenstad H, Svensson M, Cucak H, Kotarsky K, Agace WW. Differential homing mechanisms regulate regionalized effector CD8alphabeta+ T cell accumulation within the small intestine. Proc Natl Acad Sci U S A 2007;104:10122-7.
2. Edele F, Molenaar R, Gutle D, Dudda JC, Jakob T, Homey B, Mebius R, Hornef M, Martin SF. Cutting edge: instructive role of peripheral tissue cells in the imprinting of T cell homing receptor patterns. J Immunol 2008;181:3745-9.
3. Iliev ID, Mileti E, Matteoli G, Chieppa M, Rescigno M. Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. Mucosal Immunol 2009.
4. Yokota A, Takeuchi H, Maeda N, Ohoka Y, Kato C, Song SY, Iwata M. GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity. Int Immunol 2009;21:361-77.

5. Gagnon I, Duester G, Bhat PV. Kinetic analysis of mouse retinal dehydrogenase type-2 (RALDH2) for retinal substrates. Biochim Biophys Acta 2002;1596:156-62.
6. Duester G, Shean ML, McBride MS, Stewart MJ. Retinoic acid response element in the human alcohol dehydrogenase gene ADH3: implications for regulation of retinoic acid synthesis. Mol Cell Biol 1991;11:1638-46.
7. Dobbs-McAuliffe B, Zhao Q, Linney E. Feedback mechanisms regulate retinoic acid production and degradation in the zebrafish embryo. Mech Dev 2004;121:339-50.
8. Elizondo G, Corchero J, Sterneck E, Gonzalez FJ. Feedback inhibition of the retinaldehyde dehydrogenase gene ALDH1 by retinoic acid through retinoic acid receptor alpha and CCAAT/enhancer-binding protein beta. J Biol Chem 2000;275:39747-53.
9. Elizondo G, Medina-Diaz IM, Cruz R, Gonzalez FJ, Vega L. Retinoic acid modulates retinaldehyde dehydrogenase 1 gene expression through the induction of GADD153-C/EBPbeta interaction. Biochem Pharmacol 2009;77:248-57.
10. Bhat PV. Retinal dehydrogenase gene expression in stomach and small intestine of rats during postnatal development and in vitamin A deficiency. FEBS Lett 1998;426:260-2.
11. Suzuki K, Maruya M, Kawamoto S, Sitnik K, Kitamura H, Agace WW, Fagarasan S. The sensing of environmental stimuli by follicular dendritic cells promotes immunoglobulin A generation in the gut. Immunity 2010;33:71-83.
12. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, Otipoby KL, Yokota A, Takeuchi H, Ricciardi-Castagnoli P, Rajewsky K, Adams DH, von Andrian UH. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science 2006;314:1157-60.
13. Saurer L, McCullough KC, Summerfield A. In vitro induction of mucosa-type dendritic cells by all-trans retinoic Acid. J Immunol 2007;179:3504-14.
14. Tezuka H, Abe Y, Iwata M, Takeuchi H, Ishikawa H, Matsushita M, Shiohara T, Akira S, Ohteki T. Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. Nature 2007;448:929-33.
15. Zou F, Liu Y, Liu L, Wu K, Wei W, Zhu Y, Wu J. Retinoic acid activates human inducible nitric oxide synthase gene through binding of RARalpha/RXRalpha heterodimer to a novel retinoic acid response element in the promoter. Biochem Biophys Res Commun 2007;355:494-500.

16. Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity 1998;9:143-50.
17. Svensson M, Johansson-Lindbom B, F. Z, Jaenssona E, Austenaa LM, Blomhoff R, Agace WW. Retinoic acid receptor signaling levels and antigen dose regulate gut homing receptor expression on CD8+ T cells Mucosal Immunology 2008;1:38-48.
18. O'Byrne SM, Wongsiriroj N, Libien J, Vogel S, Goldberg IJ, Baehr W, Palczewski K, Blaner WS. Retinoid absorption and storage is impaired in mice lacking lecithin:retinol acyltransferase (LRAT). J Biol Chem 2005;280:35647-57.
19. Zhu Q, Egelston C, Vivekanandhan A, Uematsu S, Akira S, Klinman DM, Belyakov IM, Berzofsky JA. Toll-like receptor ligands synergize through distinct dendritic cell pathways to induce T cell responses: implications for vaccines. Proc Natl Acad Sci U S A 2008;105:16260-5.
20. Lee SH, Hu LL, Gonzalez-Navajas J, Seo GS, Shen C, Brick J, Herdman S, Varki N, Corr M, Lee J, Raz E. ERK activation drives intestinal tumorigenesis in Apc(min/+) mice. Nat Med 2010;16:665-70.
21. Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Rosemblatt M, von Andrian UH. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. Nature 2003;424:88-93.
22. Kane MA, Napoli JL. Quantification of endogenous retinoids. Methods Mol Biol 2010;652:1-54.