## 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<sup>+</sup> regulatory T cells and IgA antibody-secreting cells. We investigated whether RA functions in a positivefeedback loop in DC to induce its own synthesis. METH-**ODS:** 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.<sup>1</sup> 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  $\alpha 4\beta 7$ 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,<sup>1,2</sup> 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,<sup>3-6</sup> 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,<sup>5-7</sup> to promote differentiation of IgA antibody-secreting cells (ASC)<sup>5</sup> and to control the balance between regulatory T cells and Th<sub>17</sub> cells in the gut mucosa.<sup>8,9</sup> 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.<sup>10</sup> 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,<sup>11,12</sup> suggesting a novel crosstalk between RA- and MyD88dependent pathways.

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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 nodederived 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.



**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;  $^{*}P < .05$ ;  $^{**}P < .01$ .

### 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 ( $\alpha 4\beta 7$  and CCR9) and Foxp3<sup>+</sup> 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), which was not explained by different proportions of CD103<sup>+</sup> DC (Supplementary Figure 1C). 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 *Aldb1a2* mRNA levels in DC do not always fully correlate with their RALDH activity or gut-homing imprinting capacity. Whether this dissociation between *Aldb1a2* 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,<sup>13</sup> 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.<sup>5,7</sup> 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.<sup>14</sup> To address this shortcoming, we used mice deficient in lecithin:retinol acyltransferase (LRAT), which cannot store retinol in the liver.<sup>14</sup> 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.<sup>14</sup> 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  $\alpha 4\beta$ 7 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<sup>Int</sup> cells and IgA in the supernatant (n = 4). (D) Aldh1a2 mRNA and (E) RALDH activity in CD11c<sup>+</sup> and CD11c<sup>+</sup>CD103<sup>+</sup> DC (n = 5). (F) RALDH activity in MLN CD11c<sup>+</sup> 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 2A). In addition, MLN-DC and PP-DC from VAD mice were impaired in inducing Foxp3<sup>+</sup> T cells (Figure 2B) and IgA-ASC (Figure 2C), respectively. Moreover, MLN-DC from VAD mice showed a marked reduction in *Aldb1a2* mRNA expression (Figure 2D) and an impaired RALDH activity in total and CD103<sup>+</sup> 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 *Aldb1a2* 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 *Aldb1a2* in spleen-DC. In contrast, agonists for RXR (HX630, PA024), peroxisome proliferator-activated receptor (PPAR)- $\gamma$  (rosiglitazone), PPAR- $\beta/\delta$ (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 *Aldb1a2* mRNA. RA also induced *Aldb1a2* 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 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 guthoming receptors  $\alpha 4\beta 7$  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)<sup>17</sup> (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-



**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  $\mu$ M, 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<sup>-/-</sup> mice in fetal bovine serum–free media ± 50 nM retinol. After 4–5 days, T cells were analyzed for  $\alpha 4\beta$ 7 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<sup>-/-</sup> 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<sup>8,9</sup> RA-DC also promoted the induction of Foxp $3^+$  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.<sup>19</sup> 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 RAR $\beta$ , 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 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 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 4A). As expected, PLN-DC from control mice showed much lower levels of *Aldh1a2* mRNA and RALDH activity as compared to MLN-DC (Figure 4B and C, Supplementary Figure 4A). 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  $\mu$ g/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 4D), demonstrating that it required active RA biosynthesis.

We could not detect RA in PLN from untreated animals, even using the most sensitive methods,<sup>16</sup> 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 DR5luciferase 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<sup>+</sup> CD8 T cells in PLN and spleen upon subcutaneous immunization, reaching levels comparable to control MLN (Supplementary Figure 4D). 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 3A), 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-kB (SN50) pathways3 did not affect RA-mediated DC education (Figure 5A). However, inhibition of ERK/MAPK (U0126)<sup>3</sup> 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



**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  $\mu$ M), ERK (U0126, 10  $\mu$ M), nuclear factor- $\kappa$ B (SN50, 50  $\mu$ M), or c-Jun-N-terminal kinase (SP600125, 50  $\mu$ M). (A) DC 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 = 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  $\mu$ M) and analyzed for their luciferase activity (n = 3). (D) Wild-type mice were orally treated with the ERK inhibitor PD0325901 (25  $\mu$ g/g/dose) every other day for 6 days. After that, CD11c<sup>+</sup> MLN-DC were analyzed for CD103 expression and RALDH activity (n = 5). Mean ± SEM; "P < .05;" P < .01.

been used in vivo.<sup>15</sup> Consistent with a physiological role of ERK signaling in gut-associated DC education, treatment with PD0325901 significantly decreased RALDH activity in endogenous CD103<sup>+</sup> MLN-DC (Figure 5D), without decreasing the frequency of CD103<sup>+</sup> DC in MLN (data not shown).

Thus, ERK signaling is needed for RA-mediated DC education and is also required in vivo for endowing gutassociated 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<sup>20</sup> and that RA-mediated DC education also requires ERK signaling, we explored whether TLR signals might modulate RA effects on DC.<sup>11</sup> Consistent with this possibility, spleen-DC from mice lacking MyD88 (essential for most TLR signaling<sup>11</sup>) were impaired in their RA-mediated DC education. MyD88<sup>-/-</sup> 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<sup>-/-</sup> RA-DC (Figure 6*B*). Similarly, *Tgm2* mRNA (encoding tissue transglutaminase, a well-known RA target gene<sup>21</sup>) was not induced in MyD88<sup>-/-</sup> 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<sup>-/-</sup> and wild-type spleen-DC (data not shown).

Next, we asked whether MyD88 was required in vivo for RA-mediated DC education. MyD88<sup>-/-</sup> or wild-type mice were orally supplemented with RA. PLN-DC isolated from RA-treated MyD88<sup>-/-</sup> 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<sup>-/-</sup> mice induced lower levels of  $\alpha 4\beta 7$  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 RAinduced gene Tgm2 was lower in MyD88<sup>-/-</sup> PP-DC as compared to wild-type PP-DC, whereas Fabp4 mRNA, a PPARy-RXR target gene,10 was expressed at similar levels in MyD88<sup>-/-</sup> 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<sup>-/-</sup> 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 PPAR $\beta/\delta$ -RXR, respectively<sup>22</sup> (Supplementary Figure *5D*).



**Figure 6.** MyD88 is required for RA-mediated DC education. (*A*, *B*) Wild-type or MyD88<sup>-/-</sup> 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  $\alpha 4\beta7$  and CCR9 expression (n = 5). (*B*) Aldh1a2 (n = 4) or *Tgm2* (n = 5) mRNA expression in DC. (*C*) Wild-type or MyD88<sup>-/-</sup> mice were supplemented orally with RA (400  $\mu$ g/dose) every day for 5 days and PLN-DC were analyzed for RALDH activity (n = 7). (*D*) Spleen-DC from wild-type or MyD88<sup>-/-</sup> 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 RAR $\beta$  inhibitor LE540 (1  $\mu$ M) and then analyzed for *Aldh1a2* mRNA expression (n = 3). (*F*) Spleen-DC from UR5-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 expression (n = 3). (*F*) Spleen-DC from UR5-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.<sup>20</sup> Moreover, RA has been shown to rapidly induce ERK phosphorylation,<sup>23</sup> 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 wildtype and MyD88<sup>-/-</sup> mice. Interestingly, although RA induced ERK1/2 phosphorylation in spleen-DC, this effect was decreased on MyD88<sup>-/-</sup> DC (Supplementary Figure 5F and G). ERK phosphorylation was not affected in TLR4<sup>-/-</sup> 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 RAmediated 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<sup>-/-</sup> spleen-DC did not differ in the expression of most RAR and RXR isoforms, Rarb mRNA (encoding RAR $\beta$ ) was not detected in MyD88<sup>-/-</sup> spleen-DC (Figure 6D), suggesting that lack of RAR $\beta$ might explain, at least in part, the decreased RA-mediated education on MyD88<sup>-/-</sup> DC. In agreement with this possibility, LE540, a predominantly RAR $\beta$  inhibitor,<sup>24</sup> 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 RAR $\beta$  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<sub>3</sub>CSK<sub>4</sub> (0.5  $\mu$ g/mL), or both from day 3 of differentiation. RALDH activity was analyzed at day 6 in CD11c<sup>+</sup> cells (n = 4). FACS plots show representative results in Mo-DC from 2 donors. (*B*) Mo-DC pretreated with either RA, Pam<sub>3</sub>CSK<sub>4</sub>, or both, were washed and cultured with allogenic CD4 T cells activated with anti-CD3 plus anti-CD28 and transforming growth factor –  $\beta$ 1 (TGF- $\beta$ 1; 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<sub>3</sub>CSK<sub>4</sub> (0.5  $\mu$ g/mL), or both, washed and used to activate naive CD8 T cells. After 4 days, T cells were analyzed for their expression of  $\alpha$ 4 $\beta$ 7 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<sub>3</sub>CSK<sub>4</sub> (0.5  $\mu$ g/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  $\alpha$ 4 $\beta$ 7 (n = 3). (*E*) Spleen-DC were incubated for 24 hours in the presence of 100 nM RA, Pam<sub>3</sub>CSK<sub>4</sub> (0.5  $\mu$ g/mL), or both, or 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  $\alpha$ 4 $\beta$ 7 (n = 3). (*E*) Spleen-DC were incubated for 24 hours in the presence of 100 nM RA, Pam<sub>3</sub>CSK<sub>4</sub> (0.5  $\mu$ g/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  $\pm$  SEM; <sup>\*</sup>P < .05; <sup>\*\*\*</sup>P < .001.

TLR1/2-agonist (Pam<sub>3</sub>CSK<sub>4</sub>) 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 7*B*). Moreover, mouse spleen-DC or human Mo-DC treated with RA plus Pam<sub>3</sub>CSK<sub>4</sub> induced higher proportions of  $\alpha 4\beta 7^+$  and CCR9<sup>+</sup> T cells as compared to DC treated with either RA or Pam<sub>3</sub>CSK<sub>4</sub> 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 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  $\beta$ -carotenes (provitamin A).<sup>26</sup> 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 metabolizing enzyme  $\beta_{\beta}\beta$ -carotene-15,15'-monooxygenase 1 found in the proximal intestine as compared to ileum.<sup>27</sup> A similar proximal-to-distal expression gradient has been described for cellular retinol binding protein–II

and LRAT,<sup>28</sup> 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<sup>29</sup> (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<sup>30,31</sup> and can produce RA.<sup>7,32</sup> 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,<sup>32,33</sup> 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.<sup>17</sup> However, we did not observe impairment in *Aldh1a2* mRNA expression, RALDH activity, or gut-homing imprinting capacity in gut-associated DC from GM-CSF<sup>-/-</sup> 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.<sup>17</sup>

Similar to TLR2 stimulation,<sup>20</sup> RA-mediated DC education required signaling via ERK/MAPK. This finding is in line with previous studies showing that some RAmediated effects depend on ERK/MAPK, but not on p38/ MAPK or c-Jun-N-terminal kinase/MAPK signaling.<sup>34</sup> 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 *Gastro-enterology* at www.gastrojournal.org, and at doi:10.1053/j.gastro.2011.04.010.

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#### Conflicts of interest

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