MyD88-Dependent TLR1/2 Signals Educate Dendritic Cells with Gut-Specific Imprinting Properties

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Gut-associated dendritic cells (DC) synthesize all-*trans* retinoic acid, which is required for inducing gut-tropic lymphocytes. Gut-associated DC from MyD88^{−/−} mice, which lack most TLR signals, expressed low levels of retinal dehydrogenases (critical enzymes for all-*trans* retinoic acid biosynthesis) and were significantly impaired in their ability to induce gut-homing T cells. Pretreatment of extraintestinal DC with a TLR1/2 agonist was sufficient to induce retinal dehydrogenases and to confer these DC with the capacity to induce gut-homing lymphocytes via a mechanism dependent on MyD88 and JNK/MAPK. Moreover, gut-associated DC from TLR2^{−/−} mice, or from mice in which JNK was pharmacologically blocked, were impaired in their education to imprint gut-homing T cells, which correlated with a decreased induction of gut-tropic T cells in TLR2^{−/−} mice upon immunization. Thus, MyD88-dependent TLR2 signals are necessary and sufficient to educate DC with gut-specific imprinting properties and contribute in vivo to the generation of gut-tropic T cells. *The Journal of Immunology*, 2011, 187: 141–150.

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n addition to their well-known role as APCs, dendritic cells (DC) can modulate immune responses in a tissue-specific manner (1). DC from Peyer's patches (PP-DC) promote the induction of Th2 T cell responses and IgA Ig class-switching (1). In addition, DC from Peyer's patches, mesenteric lymph nodes, and small intestine lamina propria (gut-associated DC), but not DC from extraintestinal sites, induce a high expression of the gut-homing receptors integrin α4β7 and chemokine receptor CCR9 on mouse and human T and B cells upon activation (2–5). The se-

lective capacity of gut-associated DC to induce gut-tropic lymphocytes is explained by their ability to metabolize vitamin A (retinol) into all-*trans* retinoic acid (RA) (3, 5, 6). RA is necessary and sufficient to imprint gut-homing lymphocytes, and it also potentiates the induction of gut-homing Foxp3^{+} regulatory T cells (Treg) while reciprocally inhibiting the development of Th17 cells in vitro (7–9). Gut-associated DC, but not peripheral extraintestinal DC, express high levels of retinal dehydrogenases (RALDH), which are key enzymes for RA biosynthesis. However, it is unclear how these enzymes are specifically induced in gut-associated DC. We reasoned that bone marrow-derived uncommitted DC (or their precursors) enter the intestine from the blood and then acquire their imprinting properties in response to local differentiation signal(s) that are present in the gut mucosa. Among those environmental cues, the gut is highly exposed to pathogen-associated molecular patterns derived from the intestinal microbiota, which are recognized by pathogen recognition receptors, the best characterized of which are TLR (10). Because DC can express several combinations of TLR (11) and stimulation via TLR can differentially modulate some functional properties of DC (12), we hypothesized that TLR-mediated signals are critical for the acquisition of tissue-specific functional properties by gut-associated DC, including their capacity to produce RA, which is critical to imprint gut-homing lymphocytes.

Materials and Methods

Mice

OT-I×RAG2^{−/−}, P14×TCRα^{−/−}, C57BL/6, and C57BL/6/Thy1.1[+] mouse strains were obtained from Taconic (Germantown, NY). TLR2^{−/−}, JNK1^{−/−}, and JNK2^{−/−} mouse strains were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR6^{−/−} mice were provided by Dr. Adam Lacy-Hulbert (Massachusetts General Hospital, Boston, MA). MyD88^{−/−} TRIF^{−/−} and TLR4^{−/−} mice (13) were provided by Drs. Cathryn Nagler, Nir Hacohen, and Hans-Christian Reinecker (Massachusetts General Hospital, Boston, MA). Nalp3^{−/−} mice were provided by Dr. Jurg Tschopp (University of Lausanne, Lausanne, Switzerland). GM-CSF^{−/−} mice (14) were provided by Drs. Glenn Dranoff (Harvard Medical School, Boston, MA) and Bruce C. Trapnell (University of Cincinnati, Cincinnati, OH). DR5-luciferase mice (15) were provided by Dr. Rune Blomhoff (Cgene,

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Oslo, Norway). Mice were maintained in specific pathogen-free conditions and used in accordance with the guidelines of the Subcommittee on Research Animal Care at Massachusetts General Hospital and Harvard Medical School.

DC/T cell isolation and coculture

Mice (8–16 wk old) were injected s.c. with 5×10^6 to 10×10^6 B16 melanoma cells secreting Flt3L as described (2). After 12 to 14 d, the mice were sacrificed and peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN), and PP were digested using 250 µg/ml Liberase CI (Roche Diagnostics, Indianapolis, IN) plus 50 µg/ml DNAse-I (Roche) in IMDM (no additives) for 40 min at 37°C with mild agitation. For lamina propria (LP) DC isolation (16), the small bowels were cut into 0.5-cm pieces, placed in a 50-ml tube, and shaken at 250 rpm for 20 min at 37°C in HBSS plus 5% low-endotoxin FBS (Life Technologies, Invitrogen) plus 2 mM EDTA. Cell suspensions were passed through a strainer, and the remaining intestinal tissue was washed, placed in a 50-ml tube, and enzymatically digested as described above. Cell separations were performed at 4°C in PBS plus 2 mM EDTA plus 2% low-endotoxin FBS. DC were immunomagnetically isolated by negative selection as described (5). Negatively selected DC (85–90% CD11c$^+$) were resuspended at 10^7/ml and pulsed for 2 h at 37°C with 100 nM LCMVgp$_{33-41}$ or SIINFEKL peptides in IMDM plus 10% low-endotoxin FBS plus 50 µM 2-mercaptoethanol plus standard supplements (complete IMDM). For some experiments, negatively selected DC were sorted into CD11c$^+$CD103$^+$ and CD11c$^+$CD103$^-$ fractions (>95% purity for each DC subset; FACSVantage; BD Biosciences). Naive P14×TCRα$^{-/-}$ or OT-I×RAG2$^{-/-}$ CD8 T cells were purified from splenocytes after RBC lysis in ACK buffer by negative selection as described (2). For CFSE labeling, T cells were resuspended at 10^7 cells/ml in DMEM plus 1% FBS plus 20 mM HEPES, incubated with 2.5 µM CFSE for 20 min at 37°C, and then washed using an FBS gradient. CFSE-labeled naive CD8 T cells (1×10^5) were cocultured with peptide-pulsed DC in a 1:1 ratio in flat-bottom 96-well plates (Falcon; BD Biosciences).

Bone marrow chimeras

C57BL/6 wild-type or MyD88$^{-/-}$ mice were irradiated with 550 cGy plus 500 cGy, with 3 h between doses, and 5×10^6 total bone marrow (BM) cells from C57BL/6 wild-type or MyD88$^{-/-}$ mice were injected intravenously 3 h later, as described (17). Mice were fed with antibiotic-supplemented food (TestDiet) and were given free access to water. Chimeric mice were used for experiments 8 wk after BM transplantation. For mixed BM chimeras, irradiated wild-type mice were reconstituted with a 1:1 ratio of BM from wild-type or MyD88$^{-/-}$ mice plus BM from CD11c-DTR mice, in which DC can be specifically depleted by diphtheria toxin (DT) treatment (18). By using this strategy, we could selectively deplete wild-type DC without affecting other wild-type BM-derived cells or MyD88$^{-/-}$ DC. For DC depletion, mixed BM chimeras were inoculated i.p. with DT (Sigma-Aldrich) 8 ng/g every other day for four times.

Immunization experiments

CFSE-labeled OT-I×RAG2$^{-/-}$ T cells (5×10^6) were adoptively transferred into wild-type or MyD88$^{-/-}$ mice. Twenty-four hours later, recipients were injected i.p. with 10 mg OVA (grade VI; Sigma-Aldrich) (19) plus 1 mg alum (resuspended in 500 µl PBS) (20). In some experiments, mice were immunized with 30 mg OVA via oral gavage. Three to four days later, the mice were sacrificed, and single-cell suspensions from spleens, PLN, MLN, intraepithelial lymphocyte compartment (IEL), and LP were collected and stained for CCR9 or α4β7. The samples were analyzed in a FACSCalibur flow cytometer by gating on viable CD8$^+$ and CFSE^{int} cells.

Spleen DC conditioning

Spleen DC were isolated from Flt3L-treated C57BL/6 mice as described above. After negative selection, DC were positively selected with anti-CD11c magnetic beads (Miltenyi Biotec). DC (>98% CD11c$^+$) were pretreated for 24 h with or without different TLR ligands (InvivoGene TLR ligands kits: tlrl-kit1m2 or tlrl-kit2), washed, pulsed with antigenic peptide, and used for coculture with naive T cells, mRNA extraction, Aldefluor assay, or luciferase assay (spleen DC from DR5-luciferase mice). In some experiments, spleen DC were treated with 1 µg/ml Pam$_3$CSK$_4$ (TLR1/2 agonist) plus/minus pharmacological inhibitors for P38/MAPK (10 µM SB203580), ERK1/2/MAPK (10 µM FR180204), JNK/MAPK (50 µM SP600125), NF-κB (50 µM SN50) (21), or 1 µM LE540 retinoic acid receptor (RAR) antagonist. In some experiments, spleen DC were preincubated for 24 h with live *Escherichia coli* (K12 strain) in a 1:10 DC/bacteria ratio, washed, pulsed with antigenic peptide, and cocultured with

T cells. In other experiments, spleen DC were pretreated for 24 h with 300 µg/ml alum (77161; Thermo) and/or 500 µM ATP (FLAAS; Sigma), as described (22). For some experiments, DC were pretreated for 24 h with Pam$_3$CSK$_4$ in FBS-free media (X-vivo15; Lonza) plus/minus 50 nM retinol (R7632; Sigma-Aldrich, St. Louis, MO), as described (23).

DC immunization

Spleen DC were treated with or without 1 µg/ml Pam$_3$CSK$_4$ for 24 h, washed three times with PBS, and pulsed with LCMVgp$_{33-41}$ peptide. DC (1×10^6) were injected i.v. into Thy1.1$^+$ mice that were adoptively transferred with 4×10^6 naive CFSE-labeled P14×TCRα$^{-/-}$ CD8 T cells (Thy1.2$^+$). Four days post-DC injection, the mice were euthanized, and cell suspensions from the spleen, MLN, and PLN were analyzed by flow cytometry by gating on viable Thy1.2$^+$CD8$^+$ T cells.

In vivo JNK inhibition

Mice were treated with 50 µg/g SP600125 (Calbiochem) i.p. once a day for 5 d, as described (24). After that, the mice were euthanized and analyzed for their RALDH activity (Aldefluor staining) in MLN DC.

Statistical analysis

Unless specified otherwise, data are presented as mean ± SEM and were analyzed using GraphPad Prism Software 5.0c. Significance was set at $p < 0.05$.

Results

MyD88 is required for educating gut-associated DC with gut-homing imprinting capacity and for in vivo generation of gut-tropic T cells

In agreement with a major role of MyD88-dependent signals in gut-associated DC education, gut-associated DC isolated from mice lacking MyD88, which is an essential intracellular signaling adapter for most TLR signals (10), showed an impaired RA-synthesizing capacity, as determined by their decreased levels of *Aldh1a2* mRNA (encoding RALDH2) and *Aldh1a1* mRNA (encoding RALDH1) in DC from mesenteric lymph nodes (MLN-DC) and PP-DC, respectively (Fig. 1A). As a functional readout for RALDH enzymatic activity, we used the Aldefluor assay, which is based on a fluorescent substrate (BODIPY-aminoacetaldehyde) that upon being metabolized by RALDH enzymes accumulates in the cytoplasm of viable cells, making them fluorescent (25). In agreement with a decreased expression of *Aldh1a2* mRNA, MyD88$^{-/-}$ MLN-DC exhibited lower RALDH activity compared with wild-type MLN-DC (Fig. 1B). Importantly, consistent with a reduced ability to synthesize RA, MyD88$^{-/-}$ gut-associated DC were significantly impaired in inducing the gut-homing receptors α4β7 and CCR9 on activated TCR transgenic CD8 T cells (Fig. 1C). This was not due to a defective T cell activation, as MyD88$^{-/-}$ DC induced a similar degree of 1B11/CD43 expression [effector CD8 T cell marker (26)] and T cell proliferation (CFSE dilution) compared with wild-type DC (Supplemental Fig. 1A).

It has been shown that the gut-homing imprinting capacity is restricted to the CD103$^+$ DC subset (27), and it remained possible that CD103$^+$ gut-associated DC were specifically reduced in MyD88$^{-/-}$ mice, hence accounting for their reduced gut-homing inducing capacity. However, in agreement with a previous report showing that MyD88$^{-/-}$ mice do not show significant changes in DC subsets or in the degree of DC maturation in MLN (28), CD103$^+$ gut-associated DC were only slightly decreased in MyD88$^{-/-}$ mice (Supplemental Fig. 1B). Moreover, sorted CD103$^+$ MLN-DC from MyD88$^{-/-}$ mice expressed lower levels of *Aldh1a2* mRNA (Supplemental Fig. 1C), exhibited decreased RALDH activity (Aldefluor staining) (Supplemental Fig. 1D), and induced lower levels of CCR9 on T cells (Supplemental Fig. 1E) compared with wild-type CD103$^+$ MLN-DC. Therefore, the decreased gut-homing imprinting capacity observed in MyD88$^{-/-}$ gut-associated DC is likely explained by an intrinsic functional
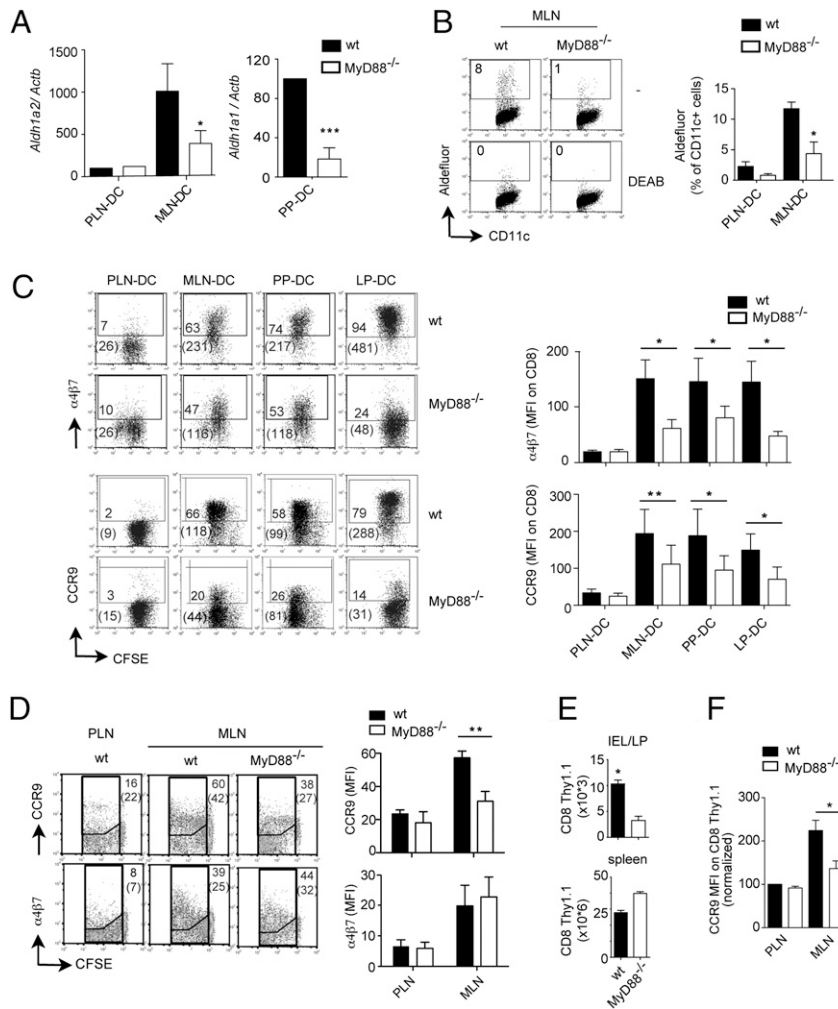
FIGURE 1. MyD88-dependent signals are necessary to confer gut-associated DC with gut-homing imprinting capacity and for in vivo generation of gut-homing T cells. *A*, TaqMan real-time PCR for *Aldh1a1* and *Aldh1a2* mRNA expression in DC (encoding for RALDH1 and RALDH2, respectively) ($n = 4$). Results were normalized with respect to *Actb* mRNA (encoding β-actin). *B*, RALDH activity (Aldefluor assay) in DC either in the absence or presence of the RALDH inhibitor DEAB ($n = 4$). *C*, Naive CFSE-labeled P14×TCRα$^{-/-}$ or OT-I×RAG2$^{-/-}$ CD8 T cells were activated with peptide-pulsed DC from peripheral lymph nodes (PLN-DC), mesenteric lymph nodes (MLN-DC), Peyer's patches (PP-DC), or small intestine lamina propria (LP-DC) from wild-type or MyD88$^{-/-}$ mice. After 4–5 d, T cells were analyzed for α4β7 and CCR9 expression by flow cytometry ($n = 6$). Numbers indicate percentage of positive cells and in parentheses the mean fluorescence intensity (MFI) of total cells. *D* and *E*, Wild-type CFSE-labeled OT-1×RAG2$^{-/-}$ CD8 T cells (Thy1.1$^+$) were adoptively transferred into wild-type or MyD88$^{-/-}$ mice (Thy1.2$^+$), which were immunized i.p. with OVA plus alum. Four days postimmunization, the mice were analyzed for (*D*) the expression of α4β7 and CCR9 on transferred activated CFSE^{Low} CD8 T cells in PLN and MLN ($n = 4$) and (*E*) the numbers of Thy1.1$^+$ CD8 T cells recovered from the small bowel LP and IEL or the spleen ($n = 6$ to 8). *F*, Same as in *D*, but the mice were immunized orally with OVA ($n = 4$). Mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

impairment in RA production in DC rather than by a reduction in the proportion of CD103$^+$ DC.

To address the possibility that some compensatory mechanisms might offset the requirement of MyD88 signaling for the generation of gut-homing T cells in vivo, we adoptively transferred OT-1/RAG2$^{-/-}$ TCR-transgenic CD8 T cells (specific for an OVA epitope) into wild-type or MyD88$^{-/-}$ mice and immunized them i.p. with OVA plus an aluminum-based adjuvant (alum), which does not require MyD88-dependent signals for its adjuvant effect (29). Although α4β7 was similarly induced on T cells activated in the MLN of wild-type or MyD88$^{-/-}$ mice, the proportion of CCR9$^+$ T cells was significantly reduced in MyD88-deficient mice (Fig. 1*D*), without a decrease in T cell proliferation (Supplemental Fig. 1*F*). Moreover, MyD88$^{-/-}$ mice exhibited a reduced number of recently activated T cells in the small bowel LP, whereas their number in the spleen was slightly increased in these mice (Fig. 1*E*). Although lower numbers of activated T cells were recruited

to the small bowel of MyD88$^{-/-}$ mice compared with that in wild-type mice, T cells that homed to the small bowel LP/IEL in MyD88$^{-/-}$ mice were selected for their high expression of CCR9 (Supplemental Fig. 1*G*). Of note, the proportion of CCR9$^+$ T cells was also reduced in MLN from MyD88$^{-/-}$ mice immunized via oral gavage (Fig. 1*F*). Thus, MyD88-dependent signals are required in vivo for educating gut-associated DC with optimal RA-synthesizing and gut-homing imprinting capacity and are also necessary for the efficient generation of gut-tropic T cells upon immunization.

Next, we asked whether gut-specific DC education requires MyD88-dependent signals acting on BM-derived cells. Our experiments using BM chimeras showed that gut-homing receptors were induced on T cells only when MLN-DC were isolated from mice in which both donor BM and recipient irradiated hosts were wild type (Fig. 2*A*, Supplemental Fig. 2*A*). Moreover, consistent with an essential role of RA synthesis in gut-homing induction by

FIGURE 2. Gut-associated DC education requires MyD88 expression in BM-derived cells and radioresistant compartment. *A*, α4β7 and CCR9 staining on CFSE-labeled P14×TCRα$^{-/-}$ CD8 T cells activated by CD11c$^+$ DC from BM chimeras in which BM from either wild-type or MyD88$^{-/-}$ donors was transplanted into wild-type or MyD88$^{-/-}$ recipient mice (*n* = 3). *B–D*, Mixed BM chimeras were generated by transplanting irradiated wild-type mice with a 1:1 ratio of BM from MyD88$^{-/-}$ mice plus BM from CD11c-DTR mice. *B* and *C*, Ten weeks later, the mice were treated with DT, which depleted CD11c$^+$ DC derived from CD11c-DTR BM. *B*, MLN-DC were analyzed for their RALDH activity (*n* = 7). *C*, Alternatively, the chimeric mice were adoptively transferred with OT1 CD8 T cells and then immunized orally with OVA. Four days later, the activated CD8 T cells were analyzed for their expression of α4β7 and CCR9 in MLN (*n* = 5). *D*, MyD88$^{-/-}$ and MyD88-sufficient CD103$^+$ MLN-DC from chimeric mice (without DT treatment) were analyzed for their RALDH activity (*n* = 2). Mean ± SEM. **p* < 0.05, ***p* < 0.01.
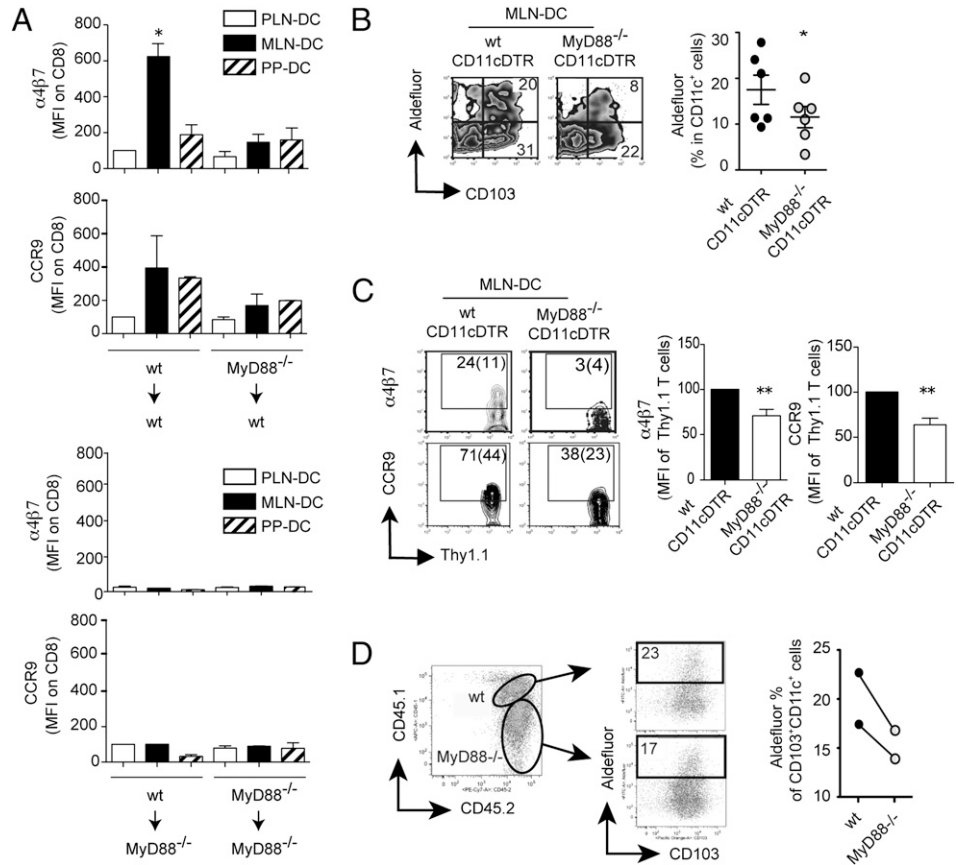

MLN-DC, their imprinting capacity correlated with *Aldh1a2* mRNA expression (Supplemental Fig. 2*B*) and RALDH activity (Supplemental Fig. 2*C*). These experiments suggest that gut-specific DC education depends on MyD88-dependent signals acting on BM-derived cells as well as in the radioresistant compartment.

To dissect whether DC need to express MyD88 to be educated in vivo or if other BM-derived cells can compensate for the lack of MyD88 in DC, we made mixed BM chimeras by reconstituting irradiated wild-type (CD45.1$^+$) mice with a 1:1 ratio of BM from MyD88$^{-/-}$ mice (CD45.2$^+$) plus BM from CD11c-DTR mice (CD45.1$^+$CD45.2$^+$), in which DC can be specifically depleted by DT treatment (18). By using this strategy, we could selectively deplete wild-type DC without affecting other wild-type BM-derived cells or MyD88$^{-/-}$ DC. Even though the remaining BM-derived cells from CD11c-DTR BM express MyD88, our results indicate that they were not sufficient to rescue the education of MyD88$^{-/-}$ MLN-DC, as the latter exhibited lower RALDH activity than MLN-DC from control mixed BM chimeras in which wild-type BM plus CD11c-DTR BM was transplanted into irradiated recipients (and also treated with DT) (Fig. 2*B*). In addition, the MyD88$^{-/-}$/CD11c-DTR mixed BM chimeras exhibited a decreased in vivo induction of α4β7$^+$ and CCR9$^+$ T cells upon oral immunization compared with control mixed BM chimeras (Fig. 2*C*). Finally, we took advantage of the congenic markers to distinguish between wild-type and MyD88$^{-/-}$ DC. We observed that MyD88$^{-/-}$ CD103$^+$ MLN-DC had lower RALDH activity when compared side by side with their MLN counterpart in the same mouse (Fig. 2*D*). Thus, our data suggest that DC need to express MyD88 to acquire optimal RA-synthesizing capacity and gut-homing imprinting ability and that the MyD88 deficiency cannot be rescued "*in trans*" by MyD88-sufficient BM-derived cells.

TLR1/2 stimulation is sufficient to educate extraintestinal DC with gut-homing imprinting capacity

Having established that MyD88-dependent signals are critical for gut-associated DC education in vivo, we asked whether stimulation via MyD88-dependent TLR might be sufficient to educate extraintestinal DC with gut-specific imprinting properties. Similar to MLN-DC and PP-DC, spleen DC expressed mRNA for most TLR, except TLR3 and TLR5 (Fig. 3*A*). To define the potential role of different TLR in educating extraintestinal DC, spleen DC were preincubated with different TLR agonists and then analyzed. Only spleen DC preincubated with the TLR1/2 agonist Pam$_3$CSK$_4$ (triacylated lipopeptide), but not with other TLR agonists, consistently upregulated *Aldh1a2* mRNA (Fig. 3*B*, Supplemental Fig. 3*A*). Accordingly, Pam$_3$CSK$_4$-stimulated spleen DC exhibited enhanced RALDH activity (Fig. 3*C*) and acquired the capacity to induce α4β7 and CCR9 on CD8 T cells (Fig. 3*D*, Supplemental Fig. 3*B*) and on CD4 T cells (Supplemental Fig. 3*C*). Because spleen DC expressed mRNA for most TLR, absence of specific TLR can only partially explain their lack of education by other TLR agonists. In fact, spleen DC express TLR4 (Supplemental Fig. 3*A* and Refs. 30–32), but the TLR4 agonist LPS was not sufficient to consistently induce *Aldh1a2* mRNA in these DC (Fig. 3*B*), which is in agreement with previous reports (25, 32). Moreover, MLN-DC from TLR4$^{-/-}$ mice were not impaired in inducing gut-homing receptors (Supplemental Fig. 3*E*). Thus, TLR4 stimulation is neither sufficient in vitro nor necessary in vivo for DC education.

Of note, the preferential induction of *Aldh1a2* mRNA by Pam$_3$CSK$_4$ correlated with an enhanced capacity to induce *Il1b* mRNA in DC (Supplemental Fig. 3*E*, 3*F*), suggesting either that Pam$_3$CSK$_4$ is more potent than other TLR agonists, that other TLR agonists/concentrations were not optimal, and/or that spleen
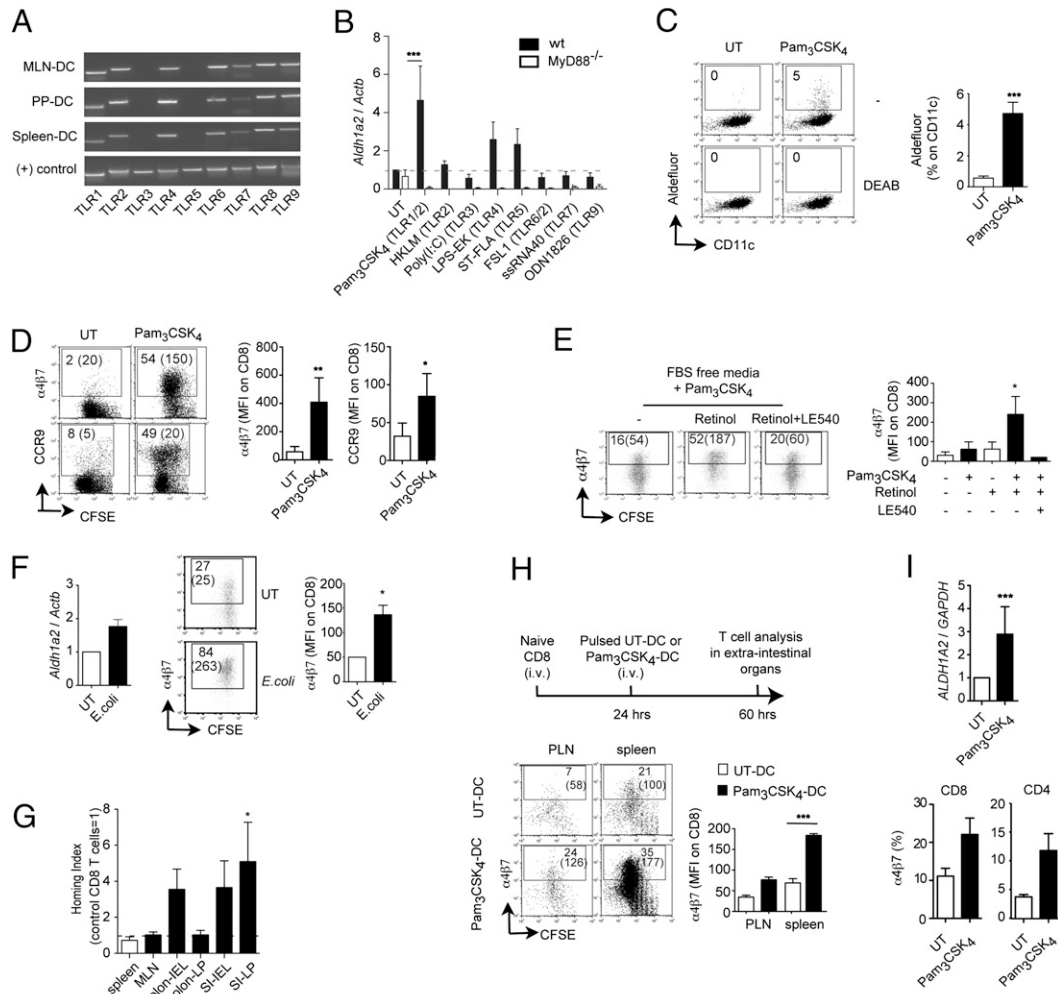
FIGURE 3. TLR1/2 stimulation is sufficient to confer extraintestinal DC with gut-homing imprinting capacity. *A*, TLR mRNA expression in freshly isolated DC. Representative of two experiments with similar results. *B*, *Aldh1a2* mRNA expression in wild-type or MyD88$^{-/-}$ spleen DC untreated or pretreated with the indicated TLR ligands (n = 3 to 6). *C*, RALDH activity in DC. Numbers indicate percentage of Aldefluor-positive cells in the indicated gate (n = 5). *D*, $\alpha 4\beta 7$ and CCR9 expression on CD8 T cells activated with spleen DC untreated or pretreated with Pam$_3$CSK$_4$ (1 μg/ml) (n = 6). Flow cytometry plots show percentage of positive cells and in parentheses mean fluorescence intensity (MFI) of total cells. *E*, $\alpha 4\beta 7$ expression on T cells activated with spleen DC untreated or pretreated with Pam$_3$CSK$_4$ in FBS-free media with or without 50 nM retinol or 1 μM LE540 (RAR antagonist) (n = 3). *F*, Spleen DC untreated or preincubated for 24 h with *E. coli* (K12 strain) and then analyzed for their expression of *Aldh1a2* mRNA (n = 2) or used to activate naive CD8 T cells. T cells were analyzed after 4 d for their expression of $\alpha 4\beta 7$ (n = 3). *G*, P14×TCRα$^{-/-}$ CD8 T cells activated with spleen DC untreated or pretreated with Pam$_3$CSK$_4$ were labeled with TRITC or CFSE, respectively, mixed in a 1:1 ratio and injected i.v. into congenic Thy-1.1$^+$ recipient mice. After 18 h, the ratio of CFSE$^+$ to TRITC$^+$ cells was analyzed in the indicated tissues. The homing index (HI) was calculated as the ratio of CFSE$^+$/TRITC$^+$ in each tissue divided by the input ratio (n = 4). Statistics were calculated using a one-sample t test versus a HI = 1 (equal migration). *H*, Thy-1.1$^+$ mice were adoptively transferred with naive CFSE-labeled P14×TCRα$^{-/-}$ CD8 T cells and then immunized i.v. with LCMV$_{gp33-41}$-pulsed spleen DC either untreated or pretreated with Pam$_3$CSK$_4$. Three days later, $\alpha 4\beta 7$ expression was analyzed on Thy1.2$^+$ CD8 T cells in PLN, MLN, and spleen (n = 3). *I*, Human monocyte-derived DC were untreated or pretreated with Pam$_3$CSK$_4$ and then analyzed for their expression of *ALDH1A2* mRNA (n = 4) or cocultured with human T cells activated with plate-bound anti-CD3 plus anti-CD28 Abs. T cells were analyzed after 5 d for their expression of $\alpha 4\beta 7$ and CCR9 (n = 3). Mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. TRITC, tetramethylrhodamine-6-isothiocyanate; UT, untreated.

DC exhibit decreased functionality for other TLR. Nonetheless, TLR-stimulated and control spleen DC induced a similar degree of T cell activation (Supplemental Fig. 3*G*), suggesting that spleen DC stimulated via different TLR do not differ significantly in their basic Ag-presenting functions compared with control spleen DC.

Consistent with a requirement of MyD88 for TLR1/2 signaling, Pam$_3$CSK$_4$ did not induce *Aldh1a2* mRNA in MyD88$^{-/-}$ spleen DC (Fig. 3*B*). A similar requirement for DC education was observed in BM-derived DC when MyD88 was knocked down using shRNA (Supplemental Fig. 3*I*). In addition, gut-homing imprinting by TLR1/2-educated DC only happened when DC and T cells were cocultured in FBS-free media (devoid of vitamin A) supplemented with retinol and it was abolished in the presence of

LE540, an antagonist of RA receptors of the RAR family (Fig. 3*E*). Moreover, RALDH2 knockdown abrogated gut-homing imprinting capacity by Pam$_3$CSK$_4$-educated DC (Supplemental Fig. 3*H*). These results indicate that TLR1/2-stimulated DC need to metabolize retinol into RA in a RALDH2-dependent manner to imprint gut-homing T cells. We also explored the possibility that TLR agonists might act directly on T cells to induce gut-homing receptors. However, none of the TLR agonists tested (including Pam$_3$CSK$_4$) significantly induced or enhanced $\alpha 4\beta 7$ or CCR9 when T cells were activated in the absence of DC (Supplemental Fig. 3*I* and data not shown).

DC in the intestinal LP can extend projections toward the intestinal lumen (33, 34) and therefore might be exposed to TLR
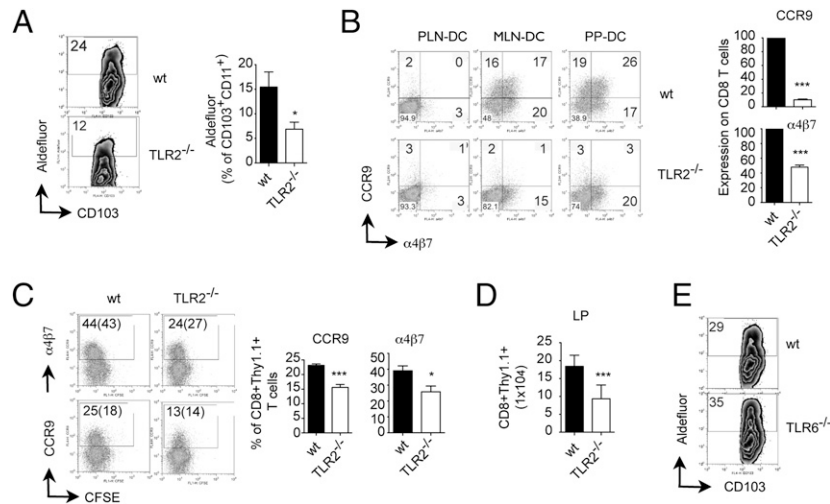
FIGURE 4. TLR2 is necessary for gut-associated DC education and for in vivo generation of gut-homing T cells. *A*, RALDH activity (Aldefluor assay) in $CD103^+$ MLN-DC from wild-type or $TLR2^{-/-}$ untreated (non-Flt3L DC expanded) mice ($n = 8$ mice/group). *B*, Naive $OT-I \times RAG2^{-/-}$ CD8 T cells were activated with peptide-pulsed PLN-DC, MLN-DC, or PP-DC from wild-type or $TLR2^{-/-}$ mice. After 4 d, T cells were analyzed for $\alpha 4\beta 7$ and CCR9 expression ($n = 4$). Numbers indicate percentage of positive cells. C and D, Wild-type CFSE-labeled $OT-1 \times RAG2^{-/-}$ CD8 T cells ($Thy1.1^+$) were adoptively transferred into wild-type or $TLR2^{-/-}$ mice ($Thy1.2^+$), which were immunized i.p. with OVA plus LPS. *C*, Four days postimmunization, the mice were analyzed for the expression of $\alpha 4\beta 7$ and CCR9 on activated $Thy1.1^+$ CD8 T cells in MLN ($n = 5$). *D*, Numbers of $Thy1.1^+$ CD8 T cells recovered from the small bowel LP and IEL 4 d postimmunization. *E*, RALDH activity in $CD103^+$ MLN-DC from wild-type or $TLR6^{-/-}$ untreated (non-Flt3L DC expanded) mice ($n = 2$ mice/group). Mean ± SEM. *$p < 0.05$, ***$p < 0.001$.

agonists from the intestinal microbiota. Therefore, to test whether bacteria-associated TLR agonists could also educate DC, we preincubated spleen DC with viable *E. coli* (K12 strain), which displays multiple TLR ligands including TLR1/2 agonists (35, 36). In agreement with this possibility, spleen DC preincubated with *E. coli* expressed *Aldh1a2* mRNA and induced higher levels of $\alpha 4\beta 7$ on T cells compared with control DC (Fig. 3*F*).

As predicted by their expression of $\alpha 4\beta 7$ and CCR9, T cells activated with Pam₃CSK₄-educated DC were bona fide gut-homing T cells and migrated significantly more to the small bowel LP compared with T cells activated with control spleen DC (Fig. 3*G*). Moreover, immunization with Pam₃CSK₄-stimulated DC induced higher levels of $\alpha 4\beta 7$-expressing T cells in vivo compared with immunization with control spleen DC (Fig. 3*H*), suggesting that ex vivo-educated DC could be used to improve gut-associated T cell responses. In fact, despite that RA has been shown to potentiate the differentiation of Treg in vitro (7, 8), Pam₃CSK₄-educated spleen DC did not induce higher levels of $Foxp3^+$ T cells than control spleen DC (Supplemental Fig. 3*J*). The lack of higher Foxp3 induction by Pam₃CSK₄-treated spleen DC might be explained by a parallel increase in the production of IL-6 (data not shown), which has been shown to antagonize Treg induction (37) and therefore might offset the effect of RA on Treg differentiation. Thus, our data suggest that TLR1/2 stimulation promotes the induction of immunogenic rather than tolerogenic DC.

Pam₃CSK₄ also induced *ALDH1A2* mRNA expression in human monocyte-derived DC and conferred these cells with gut-homing imprinting potential (Fig. 3*I*), suggesting that our findings in the murine system could be extrapolated, at least in part, to humans.

TLR2 is required for gut-associated DC education and for in vivo generation of gut-tropic T cells

Given that TLR1/2 stimulation was sufficient to confer extra-intestinal DC with gut-homing imprinting capacity ex vivo, we tested whether TLR2 has a physiological role in gut-associated DC education. In agreement with this possibility, $CD103^+$ MLN-DC

from $TLR2^{-/-}$ mice exhibited lower RALDH activity than their wild-type counterparts (Fig. 4*A*), which correlated with a decreased induction of gut-homing T cells (Fig. 4*B*). $TLR2^{-/-}$ DC did not show differences in the proportion of classical DC subsets or in their expression of maturation markers compared with their wild-type counterparts (Supplemental Fig. 4*A*).

To determine whether TLR2 is also required for the in vivo generation of gut-tropic T cells, we adoptively transferred OT-1/ $RAG2^{-/-}$ CD8 T cells into wild-type or $TLR2^{-/-}$ mice and immunized them with OVA plus LPS, as described (19). Consistent with a role of TLR2 in the generation of gut-tropic T cells in vivo, the proportions of $\alpha 4\beta 7^+$ and $CCR9^+$ CD8 T cells was significantly decreased in T cells activated in MLN from $TLR2^{-/-}$ mice (Fig. 4*C*, Supplemental Fig. 4*B*, 4*C*). Moreover, although $TLR2^{-/-}$ mice did not show a decrease in total CD8 T cells in the small bowel LP/IEL in the steady state (Supplemental Fig. 4*D*), they showed lower number of recently activated CD8 T cells in the small intestine LP compared with wild-type mice (Fig. 4*D*).

TLR2 needs to heterodimerize with either TLR1 or TLR6 to induce intracellular signaling (35). Because RALDH activity was not impaired in MLN-DC from $TLR6^{-/-}$ mice (Fig. 4*E*), our data suggest that gut-associated DC education relies on TLR1/2 rather than on TLR2/6.

TLR1/2-mediated DC education requires JNK/MAPK signaling

To obtain further mechanistic insights, we explored the role of some canonical signaling pathways in TLR1/2-mediated DC education. MAPKs have been involved in TLR-mediated effects on DC (38–40), and therefore, we assessed whether these signaling pathways are involved in TLR1/2-mediated DC education. Pharmacological inhibitors of p38/MAPK or ERK/MAPK did not impair TLR1/2-mediated DC education to induce gut-tropic T cells (Fig. 5*A*). Similarly, blocking NF-κB, a well-known downstream signaling complex in TLR signaling (40), did not affect TLR1/2-mediated DC education by Pam₃CSK₄. However, blocking JNK/MAPK significantly decreased the capacity of TLR1/2-stimulated DC to imprint $\alpha 4\beta 7$ and CCR9 on T cells (Fig. 5*A*). Moreover, JNK inhibition abrogated the induction of

FIGURE 5. Pam$_3$CSK$_4$-mediated DC education requires JNK/MAPK signaling. *A*, Spleen DC were untreated or pretreated for 24 h with 1 μg/ml Pam$_3$CSK$_4$ plus/minus pharmacological inhibitors of p38/MAPK (10 μM SB203580), ERK/MAPK (10 μM FR180204), JNK/MAPK (50 μM SP600125), or NF-κB (50 μM SN50). After that, DC were washed, pulsed with peptide, and used to activate naive CFSE-labeled OT-I×RAG2$^{-/-}$ or P14×TCRα$^{-/-}$ CD8 T cells. Four days later, T cells were analyzed for their expression of α4β7 and CCR9 (*n* = 6). *B–D*, Spleen DC were untreated or pretreated with Pam$_3$CSK$_4$ plus/minus 50 μM SP600125 (JNK inhibitor) or 1 μM LE540 (RAR antagonist). *B*, *Aldh1a2* mRNA expression in DC (*n* = 3). *C*, RALDH activity (Aldefluor staining) in DC (*n* = 3). *D*, Luciferase activity in spleen DC from DR5-luciferase mice (*n* = 3). Blockade of RA activity using 1 μM LE540 (RAR antagonist) was used as control. *E*, Spleen DC from wild-type or JNK1$^{-/-}$ mice were untreated or pretreated for 24 h with 1 μg/ml Pam$_3$CSK$_4$ plus/minus 50 μM SP600125. After that, DC were washed, pulsed with peptide, and used to activate naive CFSE-labeled OT-I×RAG2$^{-/-}$ CD8 T cells. Four days later, T cells were analyzed for their expression of α4β7 and CCR9 (*n* = 3). *F*, Wild-type mice were orally treated with 50 μg/g SP600125 i.p. once a day for 5 d. After that, CD11c$^+$ MLN-DC were analyzed for CD103 expression and RALDH activity (*n* = 4 mice/group). Mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. UT, untreated.
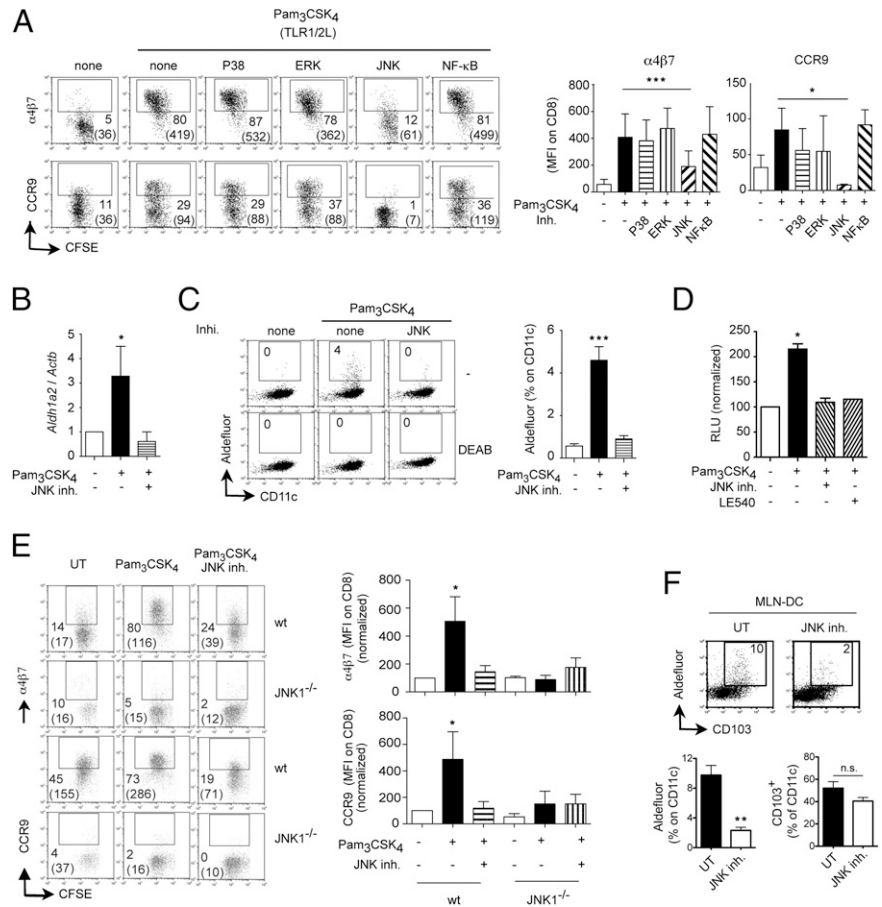
Aldh1a2 mRNA (Fig. 5*B*) and RALDH activity (Fig. 5*C*) in Pam$_3$CSK$_4$-treated spleen DC, an effect that was mirrored by a blockade of RA activity in Pam$_3$CSK$_4$-stimulated DC, as assessed in spleen DC from DR5-luciferase mice in which luciferase is controlled by a promoter with RA response elements (15) (Fig. 5*D*).

Consistent with our data using a pharmacological JNK inhibitor, spleen DC from JNK1$^{-/-}$ mice were also prevented from being educated by Pam$_3$CSK$_4$ (Fig. 5*E*). By contrast, Pam$_3$CSK$_4$ could efficiently educate JNK2$^{-/-}$ spleen DC (Supplemental Fig. 5*A*), suggesting that JNK1 but not JNK2 is required for spleen DC education. However, MLN-DC from JNK1$^{-/-}$ were not impaired in their RALDH activity or in their gut-homing imprinting capacity (data not shown), suggesting that JNK1 and JNK2 might play redundant roles in vivo. Because JNK1$^{-/-}$JNK2$^{-/-}$ double-deficient mice are embryonic lethal (41), we treated wild-type mice with the pan-JNK inhibitor SP600125, which exhibits high specificity for JNK and has been used in vivo (42). In agreement with a physiological role of JNK signaling in gut-associated DC education, mice treated with SP600125 showed a significantly lower RALDH activity (Aldefluor staining) in MLN-DC compared with control mice, without a decrease in the proportion of CD103$^+$ MLN-DC (Fig. 5*F*).

MyD88 is also required for IL-1/IL-18 signaling (13, 43), and the production of mature IL-1 or IL-18 requires inflammasome activation (44). Thus, we explored whether treatment with ATP and/or alum, which activate the inflammasome (45), was sufficient to educate spleen DC. However, these inflammasome stimuli were neither sufficient nor enhanced TLR1/2-mediated spleen DC education to imprint gut-homing T cells or to produce RA (Sup-

plemental Fig. 5*B*, 5*C*). Moreover, gut-associated DC from mice lacking the Nalp3 inflammasome, which cannot produce mature IL-1 or IL-18 (44), were not impaired in their gut-homing imprinting capacity (Supplemental Fig. 5*D*), indicating that IL-1/IL-18 signals are not required for gut-associated DC education in vivo. We also explored the role of MyD88-independent/TIR-domain–containing adapter-inducing IFN-β (TRIF)-dependent TLR signals. However, DC from TRIF$^{-/-}$ mice were not impaired in their in vivo or in vitro education to imprint gut-homing T cells (Supplemental Fig. 5*E*).

Of interest, a recent study reported that gut-associated DC from mice deficient in the common β subunit of GM-CSF/IL-3/IL-5 receptors were impaired in their gut-homing imprinting capacity, concluding that GM-CSF signals are necessary in vivo to educate gut-associated DC (25). However, gut-associated DC from GM-CSF$^{-/-}$ mice were not impaired in their capacity to synthesize RA or to induce gut-homing T cells (Supplemental Fig. 5*F*, 5*G*), suggesting that this cytokine is not essential for in vivo gut-associated DC education.

Discussion

Gut-associated DC, but not extraintestinal DC, exhibit gut-specific imprinting properties, including the capacity to generate gut-tropic lymphocytes (1). Our data support a model in which MyD88-dependent TLR1/2 signals are necessary in vivo and sufficient in vitro to optimally educate DC with gut-specific imprinting properties. TLR1/2 agonists (e.g., originated from the gut microbiota or endogenous TLR agonists) act in DC and/or DC precursors in the gut LP and induce the expression of RALDH2

(RA synthesizing capacity) via a mechanism dependent on MyD88 and JNK signaling.

A recent report showed that GM-CSF is sufficient to induce RALDH enzymes in extraintestinal DC and is also required in vivo for gut-associated DC education (25). In agreement with this study, we observed induction of *Aldh1a2* mRNA and RALDH activity in spleen DC pretreated with GM-CSF (data not shown). However, gut-associated DC from GM-CSF$^{-/-}$ mice were not impaired in their RA-synthesizing or gut-homing imprinting capacities, suggesting that GM-CSF is not essential for gut-associated DC education in vivo. The discrepancies between our results and the aforementioned study could be explained, at least in part, by the fact that we used DC from mice deficient only in GM-CSF, whereas Yokota et al. (25) isolated DC from mice deficient in the common β subunit (β-c), which is shared by GM-CSF, IL-3, and IL-5 receptor. Therefore, a combined lack of signaling via any of these receptors might potentially contribute to explain their observed phenotype.

With the purpose of obtaining a sufficient number of DC, we treated mice with Flt3L, a strategy that we as well as other groups have previously used to expand DC in all organs, including gut-associated DC (2, 5, 27, 46). Although DC obtained from Flt3L-treated mice might not be fully representative of their nonexpanded counterparts isolated from untreated mice, Flt3L expands all the classically described DC subsets in the gut, including CD103$^+$ DC (27). Importantly, we and others have shown that gut-associated DC from Flt3L-treated mice induce gut-homing receptors on T and B cells (2, 5, 27, 46), which was also observed when using gut-associated DC isolated from non-Flt3L-treated mice (4, 6, 25) or from human MLN (3, 5). Thus, DC from Flt3L-treated mice seem to maintain most of their gut-specific imprinting capacity, similar to nonexpanded murine and freshly isolated human gut-associated DC. These considerations notwithstanding, we also showed that CD103$^+$ MLN-DC from untreated MyD88$^{-/-}$ or TLR2$^{-/-}$ mice exhibited significantly lower RALDH activity compared with their counterparts from untreated wild-type mice, indicating that the requirement of MyD88 and TLR signals also applies to DC education in non-Flt3L-treated mice.

Depending on the experimental system, it has been shown that MyD88-dependent signals may be required in BM-derived DC (47), the radioresistant compartment (48), or both (49). In this regard, it has been suggested that intestinal epithelial cells (IEC) might contribute to DC education in the gut mucosa. IEC promoted gut-homing imprinting in vitro when cocultured with T cells activated with extraintestinal DC (50). Moreover, IEC conditioned extraintestinal DC ex vivo to induce Treg in a RA-dependent and TGF-β–dependent manner (51). Although the in vivo role of IEC in DC differentiation remains to be demonstrated, our data using BM chimeras suggest that gut-associated DC education depends on MyD88-dependent signals acting in BM-derived cells and also in the radioresistant compartment, such as IEC and/or stromal cells (23).

LP DC and IEC express TLR1–TLR6 (32, 52, 53), and signals via MyD88-dependent TLR, including TLR2, are necessary for maintaining intestinal epithelial homeostasis (53, 54) and for recovering the IEC compartment postirradiation (54). Therefore, lack of MyD88 in IEC might affect gut-associated DC education indirectly by disrupting normal epithelial homeostasis. Although assessing the role of MyD88 specifically in IEC would need conditional cell-specific MyD88 deletion (47), our experiments using mixed BM chimeras suggest that MyD88 needs to be expressed in DC for their optimal in vivo education and that MyD88-sufficient BM-derived cells (including DC) cannot compensate "*in trans*" for the lack of MyD88 in DC. In addition, because Nalp3$^{-/-}$ mice are not impaired in gut-associated DC education (hence excluding a role for IL-1/IL-18), our data suggest that MyD88-dependent TLR (at least in part TLR2) expressed on DC and/or DC precursors are involved in DC education by sensing TLR agonists coming from the intestinal microbiota and/or endogenous TLR agonists, which have been proposed for TLR2 (55, 56).

Notably, among MyD88-dependent TLR, only stimulation via TLR1/2 consistently educated spleen DC with gut-homing imprinting capacity. Moreover, TLR1/2 was necessary for gut-associated DC education in vivo, whereas other TLR, including TLR4, TLR6, or TRIF-dependent TLR, were not required for DC education. Although the mechanistic basis of these TLR1/2-specific effects on DC remains to be determined, it has been described that TLR2 stimulation triggers different intracellular signals compared with other MyD88-dependent TLR (38, 39, 57). In addition, it has been reported that stimulation via TLR2, but not via other MyD88-dependent TLR, confers DC with some specific functional properties, including differential cytokine secretion and a more efficient induction of effector/memory T cells (38, 39, 58).

Although MyD88$^{-/-}$ and TLR2$^{-/-}$ gut-associated DC exhibited a significant reduction in their capacity to induce α4β7 and CCR9, their impairment in gut-homing imprinting was not complete. It is possible that there are alternative pathways compensating for the lack of MyD88 or TLR2 in vivo. Stromal cells in MLN can also produce RA and induce gut-homing receptors on T cells (23, 59). Although we demonstrated that CD103$^+$ MLN-DC require MyD88 and TLR1/2 signals for their optimal education, stromal cells from MLN might not need MyD88-dependent signals for acquiring RA-synthesizing potential, hence contributing to the MyD88/TLR2-independent induction of gut-homing T cells in vivo. Moreover, despite that CCR9$^+$ T cells were significantly reduced in MLN from MyD88$^{-/-}$ mice in vivo upon immunization, α4β7 was similarly induced in wild-type or MyD88$^{-/-}$ mice. This dissociation in the induction of α4β7 and CCR9 could be due, at least in part, to a greater requirement of RA for CCR9 than for α4β7 induction on lymphocytes (3, 5, 60). A similar explanation has been proposed to explain why T cells upregulate only α4β7 but not CCR9 when the MLN is transplanted in an s.c. location (55). Thus, whereas the overall production of RA in MLN of MyD88$^{-/-}$ is apparently not sufficient to promote an optimal induction of CCR9 in vivo, it might suffice for α4β7 induction.

In addition, although our results show that the recruitment of recently activated CD8 T cells was significantly impaired in MyD88$^{-/-}$ and TLR2$^{-/-}$ mice, these mice do not exhibit an obvious defect in the numbers/proportions of T cells in the intestinal mucosa in the steady-state condition (54, 61). This is analogous to the phenotype observed in CCR9$^{-/-}$ or CCL25$^{-/-}$ mice (62, 63), which show virtually normal numbers of T cells in the intestine, but nevertheless exhibit a marked defect in the recruitment of recently activated T cells to the small bowel (4, 19, 63). Therefore, given that MyD88$^{-/-}$ and TLR2$^{-/-}$ mice are mostly impaired in acute CCR9 induction on T cells in vivo, it is not unexpected that these mice recapitulate the virtually normal gut T cell phenotype of CCR9$^{-/-}$ or CCL25$^{-/-}$ in the steady-state situation, but nonetheless they show a significantly impaired migration of recently activated T cells to the small bowel.

MAPK signaling has been involved in TLR-mediated effects on DC (38–40). Moreover, it was recently reported that zymosan (TLR2 and Dectin-1 ligand) induces RALDH expression in spleen DC via an ERK-dependent mechanism, conferring on these DC the capacity to induce Treg (64). By contrast, TLR1/2-mediated

DC education by Pam$_3$CSK$_4$ did not require ERK, but needed JNK signaling, and JNK also played a physiological role in gut-associated DC education. Our results are consistent with previous data showing that TLR1/2 signaling triggered by Pam3 (a tri-acylated lipopeptide analogue of Pam$_3$CSK$_4$) requires JNK, but not ERK or p38 signaling (40). In addition, Pam$_3$CSK$_4$-stimulated spleen DC did not promote an increase in Treg differentiation. Altogether, these results underscore the complexity of TLR signaling in which a single TLR stimulated by different agonists might trigger distinct intracellular pathways in DC with potentially different functional outcomes.

Notably, activation of the inflammasome was neither necessary nor sufficient for DC education to imprint gut-homing T cells or to produce RA. Therefore, despite promoting protective systemic immune responses, aluminum-based adjuvants, which are used in many human vaccines (29), might not be suitable for inducing effective intestinal immune responses. Thus, novel strategies for educating DC with gut-specific imprinting properties might help to improve vaccination strategies aimed at enhancing immunity in the gut mucosa.

Acknowledgments

Disclosures

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