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J Immunol 2010; 185:5915-5925; Prepublished online 13 October 2010;
doi: 10.4049/jimmunol.1001233
http://www.jimmunol.org/content/185/10/5915
Generation of Mucosal Dendritic Cells from Bone Marrow Reveals a Critical Role of Retinoic Acid

Ting Feng,* Yingzi Cong,*† Hongwei Qin,‡ Etty N. Benveniste,‡ and Charles O. Elson*†

It is unknown how dendritic cells (DCs) become specialized as mucosal DCs and maintain intestinal homeostasis. We report that a subset of bone marrow cells freshly isolated from C57BL/6 mice express the retinoic acid (RA)–synthesizing enzyme aldehyde dehydrogenase family 1, subfamily A2 (ALDH1a2) and are capable of providing RA to DC precursors in the bone marrow microenvironment. RA induced bone marrow-derived DCs to express CCR9 and ALDH1a2 and conferred upon them mucosal DC functions, including induction of Foxp3+ regulatory T cells, IgA-secreting B cells, and gut-homing molecules. This response of DCs to RA was dependent on a narrow time window and stringent dose effect. RA promoted bone marrow-derived DC production of bioactive TGF-β by inhibiting suppressor of cytokine signaling 3 expression and thereby enhancing STAT3 activation. These RA effects were evident in vivo, in that mucosal DCs from vitamin A-deficient mice had reduced mucosal DC function, namely failure to induce Foxp3+ regulatory T cells. Furthermore, MyD88 signaling enhanced RA-educated DC ALDH1a2 expression and was required for optimal TGF-β production. These data indicate that RA plays a critical role in the generation of mucosal DCs from bone marrow and in their functional activity. The Journal of Immunology, 2010, 185: 5915–5925.

Intestinal immune cells are extensively distributed throughout the intestine (1). Within the extensive network of intestinal immunity, dendritic cells (DCs) play a critical role in the decision of whether to stimulate immune regulation or activate immune responses to commensal microbiota (2). Lamina propria DCs (LP-DCs) and mesenteric lymph node DCs (MLN-DCs) have been shown to be significantly better than splenic DCs at inducing Foxp3 expression in naive T cells in the presence of exogenous TGF-β (3, 4). In addition, CD103+ DCs from both the LP and MLNs are capable of converting naive T cells into Foxp3+ regulatory T cells (Tregs) via a TGF-β and retinoic acid (RA)-dependent mechanism, whereas MLN CD103+ DCs promote the differentiation of IFN-γ-producing T cells (4–6). Peyer’s patch DCs have been implicated in B cell class switching to IgA through RA and other DC-derived signals (7). Another hallmark of intestinal CD103+ DCs is to promote the expression of the gut-homing receptors α4β7-integrin and CCR9 on responding T and B lymphocytes via RA signaling (8–10). Thus, mucosal DCs differentially regulate intestinal T cell and B cell responses in the steady state. Mucosal DCs also express pattern recognition receptors such as TLRs to recognize a broad spectrum of phylogenetically conserved microbial motifs and hence shape the innate and adaptive immune responses toward microbiota. However, the mechanisms by which DCs acquire a mucosal DC phenotype and the factors that instruct mucosal DCs to display such distinctive functional properties are unclear.

It has been shown that mice on a vitamin A-deficient diet had reductions in α4β7+ memory T cells in lymphoid organs and dramatic deficiencies of LP T cells in the small intestine (11), implicating a critical role of vitamin A and its metabolites in the homing of lymphocytes to the gut. RA, a metabolite of vitamin A, is produced by retinal dehydrogenases, such as aldehyde dehydrogenase family 1, subfamily A1 and A2 (ALDH1a1 and ALDH1a2). Consistent with their functional properties, MLN CD103+ DCs express higher levels of Aldh1a2 than their CD103− counterparts (5), and Peyer’s patch and MLN-DCs can convert vitamin A into RA in vitro (11). Accordingly, the induction of gut tropism to T cells can be partially blocked by the retinal dehydrogenase inhibitor citral (11) and can be effectively inhibited by RA receptor (RAR) inhibitors (12). These findings indicate an important role of RA in mucosal DC functions. However, whether and how RA imprints gut-homing specificity on DCs and instructs DCs to acquire the functional properties of mucosal DCs are largely unknown.

In this study, we investigated the effects of RA on mucosal DC generation. We report that bone marrow cells express ALDH1a2, and are capable of providing RA to DC precursors in the bone marrow microenvironment. RA induced bone marrow-derived DCs (BMDCs) to express CCR9 and ALDH1a2 and conferred upon them a mucosal DC phenotype and function. The effect of RA on educating BMDCs was dependent on a narrow time window and stringent dose response. RA inhibited DC expression of suppressor of cytokine signaling 3 (SOCS3), and thus enhanced STAT3 activation, which promoted bioactive TGF-β production. In cooperation with RA signaling, commensal microbiota stimulation enhanced RA-educated DC (RA-DC) ALDH1a2 expression and...
was required for maximal RA-DC TGF-β production. Together, our data indicate that RA plays a critical role in the generation of mucosal DCs from the bone marrow and in their functional activity.

Materials and Methods

Mice

C57BL/6 and C57BL/6.Foxp3 reporter mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6.CBir1 TCR transgenic (CBir1-Tg) mice (13) were bred in the Animal Facility at University of Alabama at Birmingham. Foxp3<sup>Cre</sup>CBir1-Tg mice were generated by crossing Foxp3<sup>Cre</sup> with CBir1-Tg mice. C57BL/6.Socs3<sup>fl/fl</sup> mice were kindly provided by Dr. Warren Alexander (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) (14). C57BL/6.MyD88<sup>−/−</sup> (MyD88<sup>−/−</sup>) mice were kindly provided by Dr. Suzanne Michalek (University of Alabama at Birmingham). All experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Abs and reagents

Abs against mouse phospho-STAT3<sup>tyro701</sup> and STAT3 were purchased from Cell Signaling Technology (Danvers, MA). Abs against mouse ALDH1a2 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse CD4 (RM4-5), CD11c (HL-3), IA<sup>a</sup> (AF6-120.1), CD80 (16-10A1), CD86 (GL1), CD40 (3/23), αβ (DATK32), and the Annexin V-FITC Apoptosis Detection Kit were purchased from BD Biosciences (San Diego, CA). Anti-mouse CCR9 (242Z03) was purchased from R&D Systems (Minneapolis, MN). Anti-mouse Foxp3 (FJK-16s) and intracellular staining kit were purchased from eBioscience (San Diego, CA).

Live/Dead Fixable stain was purchased from Invitrogen (Carlsbad, CA). All-trans-RA, SB-501254, and 1-methyl-t reptophan were purchased from Sigma-Aldrich (St. Louis, MO). LE135 was purchased from Tocris Bioscience (Ellisville, MO). Anti-mouse CD40 agonist Ab (FGK45) was affinity purified from hybridoma supernatants provided by Dr. Jan Anderson (Basel Institute for Immunology, Switzerland). Adenovirus expressing GFP and Cre recombinase or GFP alone under control of the CMV promoter were provided by Dr. Rosa Serra (University of Alabama at Birmingham).

Generation and activation of BMDCs

Bone marrow cells were isolated as described previously (15). Briefly, bone marrow cells were suspended at 2.5 × 10<sup>7</sup> cells/ml in complete RPMI 1640 medium containing 10% heat-inactivated FCS (Atlanta Biologicals, Atlanta, GA), 25 mM HEPES buffer, 2 mM sodium pyruvate, 50 mM 2-ME, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Cellgro Meditech, Manassas, VA). The cells were cultured in the presence of 20 ng/ml GM-CSF (R&D Systems) in 6-well plates at 37°C in 5% CO2 in humid air. RA was added at various time points as described in the text. On day 8, BMDCs were harvested and plated at 1 × 10<sup>6</sup> cells/ml per well in 24-well plates in the presence of LPS (Sigma-Aldrich), recombinant CBir1 flagellin (16), or Cpg oligodeoxynucleotide (ODN) (InvivoGen, San Diego, CA).

Chemotaxis assay

DCs (1 × 10<sup>5</sup>) were resuspended in 100 μl RPMI 1640 medium and loaded into Transwells (Corning, Lowell, MA) that were placed in 24-well plates containing 400 μl medium or medium supplemented with 10 μg/ml CCL25 or CXC11.10. After 3 h of incubation at 37°C, the migrated cells were collected and counted. The ratio of the number of DCs that migrated in the presence of chemokine versus the number of cells that migrated to the PBS control was calculated and given as the migration index.

Quantitative real-time PCR

Total RNA was extracted with TRizol reagent and followed by cDNA synthesis with Superscript reverse transcriptase (Invitrogen) as previously described (17). Quantitative PCR reactions were performed using TaqMan Gene Expression Assays for Ccr9, Aldh1a1, Aldh1a2, Socs1, Socs3, and Gapdh (Applied Biosystems, Foster City, CA) on a Bio-Rad iCycler (Bio-Rad, Hercules, CA), and all data were normalized to Gapdh mRNA expression.

Isolation of LP-DCs and spleen CD<sup>+</sup> T cells

LP leukocytes were isolated as previously described (18). DCs from LP leukocytes were further isolated by magnetic sorting (Miltenyi Biotec, Auburn, CA). CD<sup>+</sup> T cells were isolated by using anti-mouse CD4-magnetic beads (BD Biosciences).

Aldehyde dehydrogenase activity assay

Cell aldehyde dehydrogenase (ALDH) activity was determined by using the ALDEFLUOR staining kit (Stemcell Technologies, Tuukila, WA) according to the manufacturer’s instructions. Briefly, cells were resuspended at 10<sup>5</sup> cells/ml in ALDEFLUOR assay buffer containing activated ALDEFLUOR substrate with or without the ALDH inhibitor diethylamino-benzaldehyde (DEAB) and incubated at 37°C for 30 min. ALDEFLUOR-reactive cells were detected in the FITC channel.

TGF-β bioassay

As described previously (19), MFB-F11 cells are embryonic fibroblasts from Tgfb1<sup>−/−</sup> mice that are stably transfected with a reporter plasmid consisting of TGF-β responsive Smad-binding elements coupled to a secreted alkaline phosphatase reporter gene. MFB-F11 cells, kindly provided by Dr. Tony Wyse-Coray of Stanford University, were seeded at 20,000 cells per well in 96-well plates the day before assay. After incubation in 50 μl serum-free DMEM for 2 h, 50 μl supernatant of cell samples was added and incubated for 24 h. Secreted alkaline phosphatase activity shown as chemiluminescence units was measured using Great EscA-Pe SEAP Chemiluminescence kit 2.0 (Clontech, Mountain View, CA) following the manufacturer’s instructions and represents biologically active TGF-β activity.

Transient transfection of SOCS3 promoter–reporter and luciferase assay

RAW cells (American Type Culture Collection) were seeded in 12-well plates (1.5 × 10<sup>5</sup> cells/well) and transfected with the murine SOCS3 promoter (~1492 to +127 hp) using Lipofectamine 2000 (Invitrogen). Transfected cells were treated with medium, IL-6, and/or RA for 12 h, and the luciferase activity of each sample was normalized to the total protein concentration in each well. Luciferase activity from the untreated sample was arbitrarily set at 1 for calculation of fold induction.

Generation of vitamin A-modified mice

Casein-based diets were purchased from Harlan (Madison, WI) with vitamin A content modified as follows: vitamin A-deficient (VAD) diet, 0.2 IU/g (TD88407); vitamin A-control (VAC) diet, 4 IU/g (TD96007); and vitamin A-high (VAH) diet, 250 IU/g (TD96008). From the second week of pregnancy, pregnant females were fed with the respective diets and maintained throughout the pregnancy and postnatally through weaning. Progeny were weaned and maintained on the same diet.

Statistical analysis

Levels of significance were determined by Student’s t test, and p values <0.05 were considered to be statistically significant.

Results

RA educates DCs to express functional CCR9 and exhibit the phenotype of immature DCs

Expression of gut-homing receptors is likely to be the first step for DCs to reach the intestine and be further educated to acquire the full complement of mucosal DC functions. Because mucosal DCs originate in the bone marrow (20, 21), we first examined bone marrow cell ALDH1a2 expression and activity. ALDH1a2 was detected in normal C57BL/6 mouse bone marrow by Western blot, and bone marrow cells exhibited ALDH activity that was blocked by the ALDH inhibitor DEAB (Fig. 1A), indicating that the bone marrow microenvironment can provide RA to DC precursors locally.

Because it has been shown that RA induces gut-homing receptors on T cells and B cells (7, 11), the effect of RA on BMDC gut-homing receptor expression was investigated. Physiologically, serum retinol level is 0.7–2.8 μM (22), and plasma retinol levels in mice are ~1 μM (23). In tissue microenvironments in vivo, the concentration of retinol or RA between cells is unknown but is likely to be higher than that found in serum or plasma. In this sense, all data were normalized to Superscript reverse transcriptase (Invitrogen) as previously described (17).
was increased up to 1.7-fold, 5.3-fold, and 2.5-fold when RA was added from days 0, 3, and 6, respectively (Fig. 1B). Consistent with mRNA expression, control BMDCs expressed low levels (9.8%) of CCR9, but treatment with RA greatly enhanced CCR9 expression, in that 60.5% of CD11c+ DCs were CCR9+ when RA was added from day 0, 75.3% when RA was added from day 3, and 34.1% when RA was added from day 6 (Fig. 1C), indicating that the induction of gut-homing specificity via RA signaling is not restricted to lymphocytes. However, we did not observe the expression of α4β7-integrin or other chemokine receptors including CCR6, CCR7, or CXCR3 by those BMDCs (Supplemental Fig. 1B). When day 8 control BMDCs or splenic DCs were treated with RA for 48 h, RA only moderately augmented CCR9 expression in these fully developed DCs (Fig. 1D, Supplemental Fig. 2A). Based on the high-level expression of CCR9 on RA-DCs, we determined the migration capacity of control BMDCs and RA-DCs toward 10 ng/ml CCL25 or CXCL10 was analyzed and given as the migration index. Plot numbers represent the percentage of CD11c+ cells in the respective quadrants (C, D). Data are aggregate (B–E; mean ± SEM) or representative (A, C, D) of two (A) or three or more (B–E) experiments. *p < 0.05; **p < 0.01; ***p < 0.001 compared with control BMDCs.

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FIGURE 1. RA instructs BMDCs to express functional CCR9. A, ALDH1a2 expression of freshly isolated normal C57BL/6 bone marrow cells was examined by Western blot (inset), and ALDH activity was examined by incubating cells with the fluorescent ALDH substrate ALDEFLUOR with or without addition of ALDH inhibitor (DEAB) and assessed by flow cytometry. B and C, BMDCs were treated with 1 μM RA from day 0 (day0 RA-DC), day 3 (day3 RA-DC), or day 6 (day6 RA-DC) of an 8-d culture. CCR9 expression was analyzed by quantitative PCR and normalized to Gapdh mRNA expression (B) and by flow cytometry (C). D. Day 8 control BMDCs were cultured with or without 1 μM RA. CCR9 expression was determined by flow cytometry 48 h later. E. Chemotactic activity of control BMDCs and RA-DCs toward 10 ng/ml CCL25 or CXCL10 was analyzed and given as the migration index. Plot numbers represent the percentage of CD11c+ cells in the respective quadrants (C, D). Data are aggregate (B–E; mean ± SEM) or representative (A, C, D) of two (A) or three or more (B–E) experiments. *p < 0.05; **p < 0.01; ***p < 0.001 compared with control BMDCs.
FIGURE 2. Narrow time window and stringent dose response to RA for development of functional mucosal-like DCs. A, BMDCs generated as described in Fig. 1 were pulsed with CBir1 flagellin peptide and then cocultured with FACS-sorted CD4+ Foxp3+ naïve T cells from Foxp3+ CBir1-Tg mice. T cell Foxp3 expression was examined by flow cytometry after 5 d. Plots are representative of three experiments. C, The maximal induction of Foxp3 on CD4+ T cells was achieved by culturing with day 3 RA-DCs, which induced 10.5% Foxp3+ T cells, whereas control BMDCs failed to induce Foxp3+ Tregs. Collectively, these data indicate a narrow time window (RA added from day 3 of an 8-d culture) and stringent dose effect (1 μM RA) of RA induction of functional mucosal-like DCs.

To determine whether RA-DC–induced Foxp3+ T cells function like conventional Tregs, their ability to suppress naïve T cell activation was examined. Foxp3+ T cells were generated by RA-DCs or under standard Treg-polarizing conditions. FACS-sorted CD4+ Foxp3+ CBir1-Tg T cells from both conditions were cultured in triplicate wells with naïve CBir1-Tg CD4+ T cells in the presence of CBir1 flagellin peptide-pulsed splenic feeder cells. Tritiated thymidine was added for the last 18 h of a 72-h incubation period for T cell proliferation. Mean CPM of triplicates ± SEM are shown. Data are representative of three experiments. **p < 0.01; ***p < 0.001 compared with T cells were cultured alone. Tn, memory T cells; Tn, naïve T cells.

LP-DCs have been shown to induce Foxp3+ Tregs (4, 5). To determine whether RA-DCs functionally mimic LP-DCs, the capability of RA-DCs to induce Foxp3 expression in T cells was investigated. RA-DCs were generated as previously described by adding RA from day 0, day 3, or day 6 of an 8-d culture. CBir1 flagellin peptide-pulsed control BMDCs and RA-DCs were cocultured with FACS-sorted CD4+ Foxp3+ CBir1-Tg naïve T cells. T cell Foxp3 expression was examined by flow cytometry after 5 d. Plots are gated on CD4+ cells, and the percentages of Foxp3+ cells are shown (A, B). C, Naïve Foxp3+ CBir1-Tg CD4+ T cells were cultured with day 3 RA-DCs or with control BMDCs in the presence of 5 ng/ml TGF-β. CD4+ Foxp3+ T cells were sorted by FACS 5 d later. Naïve CBir1-Tg CD4+ T cells were cultured alone, with memory T cells, or with FACS-sorted Foxp3+ cells from both conditions at a ratio of 1:1 or as indicated in the presence of CBir1 flagellin peptide-pulsed splenic feeder cells. Tritiated thymidine was added for the last 18 h of a 72-h incubation period for T cell proliferation. Mean CPM of triplicates ± SEM are shown. Data are representative of three experiments. *p < 0.05; **p < 0.01; ***p < 0.001 compared with T cells were cultured alone. Tn, memory T cells; Tn, naïve T cells.

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To promote Foxp3+ Treg development, and the level of CCR9 expression. However, MLN-DCs from VAD mice lost the capability to T cells. We first bated with the fluorescent ALDH substrate ALDEFLUOR, which greater ALDH activity than that of control BMDCs when incubated with RA-DCs in the absence or presence of RA. IgA-secreting B cells via a TGF-β and RA-dependent mechanism. A, CBir1 flagellin peptide-pulsed control BMDCs and day 3 RA-DCs were cultured with naive CBir1-Tg CD4+ T cells in the presence or absence of TGF-β receptor I inhibitor SB-505124 and/or RAR antagonist LE135. Expression of Foxp3 and CCR9 by CD4+ T cells was determined by flow cytometry 5 d later. B, B220+ B cells were isolated from C57BL/6 spleen by magnetic sorting and activated with 10 μg/ml anti-mouse IgM F(ab′)2 with control BMDCs or with RA-DCs in the absence or presence of the TGF-β receptor I inhibitor SB-505124, RAR antagonist LE125, or both. IgA production by B cells was assessed by ELISA 4 d later. Bar chart represents aggregate data with mean ± SEM of three experiments. **p < 0.001 compared with the group with control BMDCs. C, MLN-DCs isolated from control (VAC) or VAD mice were pulsed with CBir1 flagellin peptide and cocultured with naive CBir1-Tg CD4+ T cells. Expression of Foxp3 and CCR9 by CD4+ T cells was determined 5 d later by flow cytometry. Data are representative of two (C) or three (A, B) experiments.

FIGURE 3. RA-DCs induce Foxp3+ Tregs and IgA-secreting B cells through a TGF-β- and RA-dependent mechanism. A, CBir1 flagellin peptide-pulsed control BMDCs and day 3 RA-DCs were cultured with naive CBir1-Tg CD4+ T cells in the presence or absence of TGF-β receptor I inhibitor SB-505124 and/or RAR antagonist LE135. Expression of Foxp3 and CCR9 by CD4+ T cells was determined by flow cytometry 5 d later. B, B220+ B cells were isolated from C57BL/6 spleen by magnetic sorting and activated with 10 μg/ml anti-mouse IgM F(ab′)2 with control BMDCs or with RA-DCs in the absence or presence of the TGF-β receptor I inhibitor SB-505124, RAR antagonist LE125, or both. IgA production by B cells was assessed by ELISA 4 d later. Bar chart represents aggregate data with mean ± SEM of three experiments. **p < 0.001 compared with the group with control BMDCs. C, MLN-DCs isolated from control (VAC) or VAD mice were pulsed with CBir1 flagellin peptide and cocultured with naive CBir1-Tg CD4+ T cells. Expression of Foxp3 and CCR9 by CD4+ T cells was determined 5 d later by flow cytometry. Data are representative of two (C) or three (A, B) experiments.

RA upregulates DC ALDH1a2 expression and induces bioactive TGF-β production

Because RA-DCs promoted the development of Foxp3+ Tregs and IgA-secreting B cells via a TGF-β- and RA-dependent mechanism (Fig. 3A, 3B), we asked whether RA-DCs, similar to mucosal DCs, provide a source of RA and TGF-β to T cells. We first examined the gene expression levels of Aldh1a1 and Aldh1a2 in both control BMDCs and RA-DCs. Aldh1a1 was not detected in either type of DC (data not shown), whereas treatment with RA induced substantial expression of Aldh1a2 mRNA (Fig. 4A) and protein (Fig. 4B) in RA-DCs. Accordingly, RA-DCs exhibited greater ALDH activity than that of control BMDCs when incubated with the fluorescent ALDH substrate ALDEFLUOR, which was blocked by the inhibitor DEAB (Fig. 4C). RA also regulated Aldh1a2 expression in intestinal DCs in vivo; mice fed with a VAH diet expressed a significantly higher level of Aldh1a2 than that of control (VAC) mice, whereas VAD mice showed a profound defect in Aldh1a2 mRNA expression. Restoration of vitamin A in the VAD mice by switching from the VAD diet to a normal diet restored Aldh1a2 expression levels comparable with those of the control mice (Fig. 4D). The dependence of mucosal Aldh1a2 expression on dietary vitamin A levels was accompanied by a concomitant decrease of Aldh1a2 expression in bone marrow cells from VAD mice (Supplemental Fig. 3). These data indicate that ALDH1a2 is robustly induced by RA in DCs both in vitro and in vivo.

To quantify RA-DC TGF-β production, a reporter cell line MFB-F11 (19) was used. Neither control BMDCs nor RA-DCs secreted any detectable levels of biologically active TGF-β without stimulation (Fig. 4E). However, upon stimulation with agonistic anti-CD40 Ab, RA-DCs secreted high levels of bioactive TGF-β. It has been shown that apoptotic cells also secrete TGF-β (26, 27). To exclude this possibility, apoptosis of DCs was analyzed by staining with 7-aminoactinomycin D and annexin V Ab. There was no difference in apoptotic cells between control BMDCs and RA-DCs (Fig. 4F). Collectively, these data demonstrate that RA endows DC precursors with mucosal DC functionality by inducing DCs to express ALDH1a2 and to produce bioactively active TGF-β.

RA stimulates TGF-β production by suppressing DC SOCS3 expression

We next explored the mechanism by which RA induces DCs to produce bioactive TGF-β. Activated STAT3 has been reported to directly upregulate TGF-β1 promoter activity (28, 29), and SOCS3 is known to negatively regulate STAT3 activation (30). To
compare Sox3 gene expression in control BMDCs and RA-DCs, we performed real-time PCR at serial time points. SOCS3 is an inducible protein and is not constitutively expressed in most types of cells, including DCs. IL-6–induced expression of Sox3 peaked at 30 min and subsided to baseline after 8 h in both DC groups. However, RA-DCs expressed a significantly lower amount of Sox3, but not Sox1, mRNA than that of control BMDCs at all time points (Fig. 5A). Analysis of SOCS3 promoter activity revealed a repressive role of RA on IL-6–induced SOCS3 expression (Fig. 5B). We then examined STAT3 activation status in RA-DCs by Western blot analysis. Consistent with low levels of SOCS3 expression, STAT3 activation in RA-DCs was enhanced and prolonged compared with that in control BMDCs in response to IL-6 or LPS stimulation (Fig. 5C).

It has also been shown that loss of SOCS3 expression promotes STAT3-mediated TGF-β production in various types of cells, including DCs (28, 29, 31). To determine the effect of SOCS3 expression on TGF-β production in RA-DCs, we first deleted the Sox3 gene in BMDCs by treating Sox3flox/flox BMDCs with Cre-expressing adenovirus. Sox3flox/flox BMDCs were also treated with control GFP-expressing adenovirus. Deletion of the Sox3 gene was confirmed by genotyping and real-time PCR (Fig. 5D). When treated with anti-CD40 agonist Ab or various TLR ligands, SOCS3−/− BMDCs, similar to RA-DCs, produced a substantial amount of bioactive TGF-β (Fig. 5E). Accordingly, SOCS3−/− BMDCs promoted de novo generation of Foxp3+ Tregs, which was completely inhibited by the TGF-β receptor I inhibitor SB-505124 (Fig. 5F). Collectively, our data demonstrate
that RA promotes DC TGF-β production by suppressing SOCS3 expression, which releases the inhibition of SOCS3 on STAT3 activation.

**TLR-MyD88 signaling is required for optimal RA-DC function**

Commensal bacteria have a great impact on the mucosal immune system (32). Once gut-homing receptor-expressing DCs migrate to the intestine, they are exposed to a diverse array of microbiota ligands that can stimulate TLR signaling, mostly through the adapter protein MyD88. To unravel the role of microbial ligands in mucosal DC function, we first compared the function of MyD88−/− mucosal DCs with that of wild-type mucosal DCs. As shown in Fig. 6A, freshly isolated mucosal DCs from MyD88−/− mice induced fewer Foxp3+ Tregs than that of DCs from wild-type mice. Similarly, we observed less induction of T cell Foxp3 expression by RA-DCs from MyD88−/− mice compared with that of wild-type RA-DCs (Fig. 6B). This could be due to a maturation defect in bone marrow cells of MyD88−/− due to lack of TLR signaling by endogenous ligands or could be due to low levels of TLR ligands present in culture medium and/or FBS (33) stimulating wild-type but not MyD88−/− bone marrow cells. To determine whether mucosal DCs need to be activated by TLR-
MyD88 signaling to produce maximal amounts of TGF-β, we measured bioactive TGF-β production by wild-type RA-DCs upon stimulation by different TLR ligands. LPS, full-length CBir1 flagellin, and CpG ODN all stimulated TGF-β production in wild-type RA-DCs (Fig. 6C). As expected, MyD882/2 RA-DCs did not produce detectable levels of bioactive TGF-β in response to stimulation with LPS, flagellin, or CpG ODN. In contrast, MyD882/2 RA-DCs did produce bioactive TGF-β in response to anti-CD40 agonist Ab at a comparable level to that of wild-type RA-DCs (Fig. 6C). These data indicate that TLR-MyD88 signaling is required for optimal TGF-β production by mucosal DCs or RA-DCs and hence the induction of Foxp3 expression in T cells.

**Discussion**

Intestinal mucosal DCs have distinct phenotypical and functional properties that allow them to differentially regulate host immune responses toward microbiota and pathogens, which is pivotal to the maintenance of intestinal immune homeostasis (2, 34). Several functional aspects of mucosal DCs have been reported, including imprinting lymphocytes with gut-homing tropism, promoting IgA-producing B cells, and inducing the differentiation of Tregs that are involved in tolerance to soluble oral Ags and commensal bacteria (2). Recent reports demonstrated that common macrophage and DC precursors can give rise to both CD103+ and CD103− DCs, whereas DC precursors or monocytes can differentiate only into CD103+ or CD103− DCs, respectively (20,
Consistent with previous observations (35, 36), our data show that functionally active ALDH1a2 was present in the bone marrow, suggesting that RA can be produced in bone marrow and affect DC differentiation in that microenvironment. It has been shown that RA imparts gut tropism to T and B lymphocytes by inducing αβ2-integrin and CCR9 expression in these cells (7, 11). We found that the addition of RA to BMDC cultures greatly promoted BMDC CCR9 expression, and such RA-DCs migrated toward the CCR9 ligand CCL25, which is constitutively expressed in the small intestine (37, 38), indicating that RA can also imprint DCs with gut-homing specificity. When cultured with naïve CD4+ T cells, RA-DCs induced αβ2 and CCR9 expression in T cells and promoted naïve T cell conversion to Foxp3+ Tregs. RA-DCs also promoted B cell IgA production. Mucosal DC induction of T cell Foxp3 expression was impaired in VAD mice, indicating that vitamin A and its metabolite RA modulate mucosal DC development and function in vivo. Collectively, our data support the notion that mucosal DCs originate in the bone marrow, and RA, produced locally by bone marrow cells, instructs DCs to initiate differentiation to mucosal DCs both phenotypically and functionally.

Notably, the effect of RA on differentiating mucosal-like DCs occurred within a narrow time window and stringent dose response. When a high dose (1 μM) of RA was added from day 3 of an 8-d BMDC culture, it induced DCs to acquire mucosal DC functions with maximum capability. RA did not confer upon fully differentiated BMDCs or resident splenic DCs the ability to induce T cell Foxp3 expression. This time window and dose effect of RA on BMDC development into mucosal-like DCs in vitro implies that in the bone marrow, RA induction of mucosal-like DCs is tightly regulated spatially and temporally. However, how this time and dose control is related to ALDH1a2 expression is currently unknown. Whether SOCS3 expression has to be kept low, and/or ALDH1a2 expression needs to be increased and maintained high, or if any other gene is involved is under active investigation. Bone marrow cells contain heterogeneous populations that may represent different stages of development. How this narrow time window and stringent dose response of RA applies to in vivo DC development is an intriguing future investigation. We speculate that DC precursors will develop into mucosal DCs in selected niches within the bone marrow. The identity of niche ALDH-expressing bone marrow cells is unknown at present but is the subject of further inquiry.

RA did not induce DCs to express CD103, a hallmark of a subpopulation of mucosal DCs, even though RA-DCs produced TGF-β, which has been shown to stimulate CD103 expression on T cells (39). The lack of CD103 expression by RA-DCs is not due to low production of TGF-β by RA-DCs, because addition of exogenous TGF-β alone or together with RA in BMDC culture did not induce DC CD103 expression either (data not shown), suggesting that other factors, such as epithelial cell-derived signals, may contribute to the induction of CD103 in mucosal DCs (40). Of note, despite the lack of CD103 expression, RA-DCs still possess mucosal DC functions because they induced Foxp3+ Tregs as is shown in RA-DCs and IgA-secreting B cells, indicating that CD103 is not obligatory for these mucosal DC functions (41).

RA and TGF-β are cofactors in mucosal DC function. Our data demonstrate that blockade of TGF-β signaling greatly inhibited RA-DC induction of T cell Foxp3 expression. The RAR antagonist partially downregulated Foxp3 but almost completely abolished CCR9 expression. These data indicate that both RA-DC–produced TGF-β and RA are responsible for the induction of T cell Foxp3 expression by RA-DCs, whereas induction of CCR9 expression relies only on RA production.

STAT3 has been reported to be important in mucosal immune regulation. However, STAT3 in T cells and innate cells seems to function differently in terms of controlling mucosal immune responses. Loss of STAT3 in T cells abrogates their ability to induce chronic intestinal inflammation due to their inability to develop into Th17 cells (42), whereas STAT3 deficiency in innate cells results in intestinal inflammation (43, 44). STAT3 can bind to the TGF-β1 promoter and elevate its activity (28). Constitutive expression of activated STAT3 in DCs induces TGF-β, whereas a dominant negative form of STAT3 suppresses TGF-β production.
(28). SOCS3 regulates immune responses through inhibition of STAT3 activation (45, 46). Thus, STAT3 positively and SOCS3 negatively regulates the production of TGF-β by DCs (29, 31). DCs from SOCS3−/− mice have constitutive activation of STAT3 and produce higher levels of TGF-β than those of wild-type DCs (31), probably through enhanced STAT3 recruitment to the promoter of TGF-β (28). Our data demonstrate decreased levels of SOCS3 expression and an increased and prolonged activation status of STAT3 in RA-DCs. RA directly or indirectly suppressed SoCs3 gene promoter activity and hence DC SOCS3 expression. Therefore, RA promotes DC TGF-β production by suppressing SOCS3 expression, which releases the inhibition of SOCS3 on STAT3 activation. This is at least one mechanism by which RA induces DCs to produce TGF-β and thus instructs mucosal DC development.

Commenzial microbiota critically shapes the mucosal immune system, and some organisms may have a greater impact on mucosal immunity than others (47–50). In regard to the effect of gut microbiota on the mucosal immune system, and some organisms may have a greater impact on mucosal immunity than others (47–50). In regard to the effect of gut microbiota on the mucosal immune system, and some organisms may have a greater impact on mucosal immunity than others (47–50).

In summary, our data are consistent with a “multiple-hit model” for RA-mediated mucosal DC generation: mucosal DC precursors encounter RA at the right time in the right microenvironmental niche in the bone marrow, where RA induces mucosal DC precursors to express CCR9 and ALDH1a2. CCR9α and ALDH1α2, which are induced in response to retinoic acid, are expressed on DCs in the small intestine.

Acknowledgments

We thank Dr. Craig Maynard for assisting in the experiments with vitamin A-modified diet-fed mice. Dr. Donald Buchsbaum for offering the ALDEFLUOR staining kit, and Dr. Yasmine Belkaid (National Institute of Allergy and Infectious Diseases, National Institutes of Health) and Dr. Rodrigo Mora (Harvard University, Boston, MA) for providing certain bone marrow samples. We also thank Enid Keyser for assistance in cell sorting by flow cytometry.

Disclosures

The authors have no financial conflicts of interest.

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