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Retinoic acid promotes the development of Arg1-expressing dendritic cells for the regulation of T-cell differentiation

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Summary

Arginase I (Arg1), an enzyme expressed by many cell types including myeloid cells, can regulate immune responses. Expression of Arg1 in myeloid cells is regulated by a number of cytokines and tissue factors that influence cell development and activation. Retinoic acid, produced from vitamin A, regulates the homing and differentiation of lymphocytes and plays important roles in the regulation of immunity and immune tolerance. We report here that optimal expression of Arg1 in dendritic cells requires retinoic acid. Induction of Arg1 by retinoic acid is directly mediated by retinoic acid-responsive elements in the 5' non-coding region of the *Arg1* gene. Arg1, produced by dendritic cells in response to retinoic acid, promotes the generation of FoxP3⁺ regulatory T cells. Importantly, blocking the retinoic acid receptor makes dendritic cells hypo-responsive to known inducers of Arg1 such as IL-4 and GM-CSF in Arg1 expression. We found that intestinal CD103⁺ dendritic cells that are known to produce retinoic acid highly express Arg1. Our results establish retinoic acid as a key signal in expression of Arg1 in dendritic cells.

Keywords

arginase; dendritic cells; retinoic acid

Introduction

Catabolism of arginine by nitric oxide synthases and arginases plays a variety of important roles in the immune system. Mammalian arginases, expressed as two isozymes (Arg1 and Arg2), hydrolyze arginine to produce urea and ornithine [1]. Genetic deletion studies revealed that Arg1, but not Arg2, is required for survival of mice [2, 3]. In the immune system, Arg1 is implicated in the downregulation of immune responses [4-6]. While Arg1 is primarily expressed by macrophages and myeloid-derived suppressor cells within the immune system, dendritic cells (DCs) also express Arg1 [7]. Expression of Arg1 in myeloid cells is induced by Th2 cytokines such as IL-4 and IL-13 [7-9]. Other factors that promote the production of Arg1 include TGF- β , GM-CSF, prostaglandin E, catecholamine, and toll-like receptor ligands [8, 10-12]. Arg1 antagonizes the anti-microbial effect of nitric oxide

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synthase (NOS) 2 and suppresses T-cell activation by depleting arginine [13]. Some bacterial pathogens exploit this property of arginases to evade NO-dependent killing [12].

Retinoic acid (RA) plays important roles in the immune system. RA is required for the differentiation of myeloid cells [14, 15]. RA is particularly important for promoting mucosal immunity by inducing gut-homing effector T cells and IgA-producing B cells [16-18]. Moreover, RA at low concentrations is required for general effector T-cell responses in non-mucosal tissues [19, 20]. RA is produced from retinol (vitamin A), which is absorbed in the intestine by epithelial cells and antigen presenting cells [16, 21]. RA not only plays an important role in migration of effector lymphocytes to the intestine but also promotes the generation of gut FoxP3⁺ regulatory T cells [22-24]. RA elicits TGF- β -secreting DCs and gut-homing receptor (CCR9 and α 4 β 7)-expressing T cells [25]. Moreover, RA induces the expression of CCR9 and ALDH1a2 (RALDH2) in DCs [26] and induces tolerogenic DCs with a reduced ability to produce inflammatory cytokines [27].

We report that RA generates DCs expressing Arg1. RA induces Arg1 expression by bone marrow (BM)-derived DCs and enhances the Arg1 induction activity of IL-4. RA receptor (RAR) blockade suppresses Arg1 expression by DCs. RA-induced Arg1 plays an active role in induction of FoxP3⁺ T regulatory (Treg) cells by dendritic cells. We also provide a potential molecular mechanism behind the regulation of the *Arg1* gene by RA.

Results

RA induces Arg1 expression in DCs

RA has important regulatory effects on myeloid cells [28], and expression of Arg1 by DCs has been documented a decade ago [7]. The factor(s) that regulates the expression of Arg1 by DCs and the function of the Arg1 produced by DCs remains unclear. To determine if RA is a regulatory factor in expression of Arg1 by DCs, we generated BM-DCs with GM-CSF in the presence or absence of RA (RA-DCs or control DCs). Alternatively, Ro41-5253 (abbreviated to “Ro41”) was used to block the effect of RA during DC generation in vitro (termed “Ro-DCs”). This was necessary to block the effect of RA normally present at low levels in the culture medium containing fetal bovine serum. On average, 95-98% of these cells were CD11c⁺ after culture (not shown). Control DCs, RA-DCs, and Ro41-DCs were comparable in cell viability (Supporting Information Fig. 1), overall antigen phenotype before and after activation with LPS (Supporting Information Fig. 2A), and priming activity for T-cell proliferation (Supporting Information Fig. 2B). We performed a genome-wide microarray study on these cells and found that Arg1 was one of the most highly up-regulated genes by RA in DCs (Fig. 1A and Supporting Information Fig. 3). Other genes highly up or down-regulated in DC by RA or Ro-41 are shown in Figure 1A and Supporting Information Fig. 3. We found that expression of Arg1 mRNA and protein was highly induced in RA-DCs compared with that in control or Ro-DCs (Fig. 1B and C). We generated BM-DCs also with FLT3L and examined if RA induces Arg1 in these DCs (Fig. 1D). Expression of Arg1 mRNA and protein was induced also in FLT3L-induced DCs (Fig. 1D and Supporting Information Fig. 4). When the activity of cellular arginase was examined based on urea production, the arginase activity was low in the DCs cultured with Ro41 (Fig. 1E). In contrast, arginase activity was high in the DCs cultured with RA. The difference of Arg1 expression or activity between RA-DCs and Ro41-DCs was maintained even after activation of dendritic cells with LPS (Fig 1B and E).

In contrast, Arg2 expression was not significantly changed by RA or Ro41 (Fig. 1F). RA also induced SLC7A2/CAT2B, which is a high capacity cationic solute carrier involved in transportation of arginine and related amino acids [29]. Expression of CAT2B mRNA was increased by RA and LPS and suppressed by Ro41 (Fig. 1F). As reported [30], expression of

RALDH2 was increased by RA in DCs. Expression of other RALDH enzymes such as RALDH1 and RALDH3 was extremely low in BM-DCs, and RA did not induce these enzymes (not shown).

RA can be produced by RALDH1 and RALDH2 expressed by gut epithelial cells and dendritic cells in the small intestine [16, 31, 32]. Therefore, a likely tissue where Arg1 is induced by RA is the intestine. To determine this possibility, we examined the expression of Arg1 by CD11c⁺ DCs isolated from the small intestinal lamina propria of mice. The mice were injected with FLT3L-expressing B16 cells [33] to obtain sufficient numbers of CD11c⁺ DCs for western blotting. We found that the CD11c⁺ cells isolated from the mouse intestine had high expression of Arg1 protein (Fig. 2A). In contrast, expression of Arg1 protein by the CD11c⁺ DCs isolated from the spleen of the same mice was not readily detectable.

We further examined Arg1 expression by CD103⁺ and CD103⁻ CD11c⁺ DC subsets in the small intestine (Fig 2B). We found that small intestinal lamina propria CD103^{high} CD11c⁺ DCs expressed Arg1 at levels higher than CD103^{low} CD11c⁺ DCs. CD11b^{high} F4/80⁺ macrophages isolated from the small intestinal lamina propria highly expressed Arg1 but the expression level was somewhat lower than that of the CD103⁺ DCs (Fig 2B).

RA and IL-4 cooperatively induce Arg1 expression

An intriguing question is if RA can cooperate with known Arg1-inducing factors such as IL-4. We confirmed that IL-4 can induce Arg1 expression on its own as reported but can further induce the expression together with exogenous RA (Fig. 3A and B). Moreover, Ro41 suppressed the effect of IL-4 in induction of Arg1 expression, suggesting that RA and RA receptors are required for the optimal response of DCs to IL-4 in expression of Arg1 at mRNA and protein levels.

We next examined the expression levels of Arg1 in myeloid cells developed from BM cells cultured with M-CSF, which is known to promote macrophage differentiation [8]. RA had a positive effect on expression of Arg1 in these cells (Fig. 3C). However, the induction level of Arg1 in these cells was very low compared with that in GM-CSF-activated cells. Arg1 expression at the protein level in RA and M-CSF-stimulated myeloid cells was not readily detected (Fig. 3D).

Roles of RAR binding sites in regulation of the *Arg1* gene promoter

Because of the roles of RARs in mediating the RA signal, we examined the expression of RAR α , RAR β and RAR γ in RA-DCs and Ro-DCs Fig. 4A. All RAR isotypes were highly expressed in RA DCs compared with those of other DCs. The RAR α mRNA was expressed at a level higher than those of other RARs in RA-DCs.

We, next, examined if RA regulates the *Arg1* promoter activity. We utilized a reporter system that expresses a luciferase under the regulation of the 4.8 kb *Arg1* promoter in the 5' upstream region of the gene [34]. We transfected this reporter construct into BM-DCs, treated the cells with RA and/or IL-4, and examined the level of luciferase expression. With this reporter system, RA had a positive effect on activation of the 4.8 kb *Arg1* promoter region (Fig. 4B). IL-4 increased the promoter activity as well. Interestingly, RA and IL-4 cooperatively enhanced the promoter activity.

RA can regulate gene expression through RAR-RXR heterodimeric nuclear receptors, which bind RAR response elements (RAREs) for transcriptional regulation of a number of genes [35]. When we examined the presence of RAR binding sites on the *Arg1* gene, we found four potential RAREs (RARE A, B, C, and D) (Fig. 4C). We performed a chromatin immunoprecipitation assay to assess RAR binding to the RAREs in the RA-treated DCs.

This assay revealed positive RAR binding to the RAREs at -2747 and -961/972 (Fig. 4D). The RAR binding to the RAREs was partially decreased with RAR antagonists (Ro41 and LE540).

We introduced null mutations into the three RAREs and examined if these RAREs are required for the RA-induced regulation of the *Arg1* promoter activity (Fig. 4E). Mutations in the three RARE sites abolished most of the RA-induced promoter activity (Fig. 4E). The mutations decreased the *Arg1* promoter activity induced in all conditions. This suggests that RA, typically present in the culture medium at low concentrations (~1 nM) due to the RA originating from fetal bovine serum, plays a role in supporting the basic and induced activities of the *Arg1* promoter.

RA-induced Arg1 promotes the induction of FoxP3⁺ T cells by DCs

RA-DCs and DCs isolated from the intestine are efficient inducers of Treg cells [26, 36, 37]. We hypothesized that Arg1 would play a role in this process. We examined the activity of RA-DCs, control DCs, and Ro-DCs in induction of naïve T-cell differentiation into FoxP3⁺ T cells. Naïve T cells from DO11.10 Rag2 (-/-) mice were activated with OVA₃₂₃₋₃₃₉ peptide-pulsed control, RA- or Ro-DCs (Fig. 5A). RA-DCs were most efficient in induction of FoxP3⁺ T cells (Fig. 5A), whereas Ro-DCs were unable to induce FoxP3⁺ T cells. We used arginase inhibitors such as BEC [(S)-(2-Boronoethyl)-L-cysteine] and Nor-NOHA [N-hydroxy-nor-L-arginine] to assess the function of Arg1 in this process. RA-DC-dependent induction of FoxP3⁺ T cells was inhibited by ~50% in the presence of the arginase inhibitors (Fig. 5B). The Treg-cell induction by control DCs was also suppressed by the inhibitors. The arginase inhibitors did not promote the death of BM DCs at the concentrations employed in this study (Supporting Information Fig. 5). These results suggest that RA-induced Arg1 promotes the function of DCs in inducing FoxP3⁺ T cells.

RA-induced cell-free Arg1 can promote Treg-cell generation

Arg1 can be released from activated macrophages [38]. Also, it is plausible that active Arg1 would be leaked out of dying cells and affect the local metabolic milieu. We examined the presence of Arg1 in the conditioned medium of BM-DCs. It was greatly increased in the conditioned medium of RA-DCs but barely detectable in the conditioned medium of control DCs (Fig. 5C). Arg1 was not detected in the conditioned medium of Ro-DCs. This difference would not be due to differential cell death because the viability of the DC populations was comparable to each other (Supporting Information Fig. 1). The conditioned medium of RA-DCs (2-day old; free of exogenous RA) when added to the culture of naïve T cells had an enhancing effect on generation of induced Treg cells (Fig. 5D). This Treg-inducing activity of the conditioned media was suppressed by arginase inhibitors (Fig. 5D).

While the experiment with arginase inhibitors provided useful information regarding the role of Arg1 in induction of Treg cells, the inhibitors suppress both Arg1 and Arg2 and thus cannot rule out the involvement of Arg2. To address this issue, we utilized BM-DCs derived from Arg1-deficient mice, which were confirmed to lack Arg1 expression (Fig. 6A). Arg1-deficient mice develop severe hyperammonemia and die around days 10-14 [2]. Unlike their wild type counterparts, the Arg1(-/-) RA-DCs were not able to efficiently induce FoxP3⁺ T cells (Fig. 6B). This information supports the positive role of Arg1 produced by BM-DCs in regulation of T-cell differentiation into FoxP3⁺ T cells.

Th17 cells are effector T cells induced by TGF- β 1 in an inflammatory cytokine milieu including IL-6 and IL-23. We examined if Arg1 has any effects on T-cell differentiation into Th17 cells. RA-DCs were not effective in inducing Th17 cells, whereas control and Ro-DCs were more efficient in inducing Th17 cells (Supporting Information Fig. 6A). Arg1

inhibitors somewhat suppressed the Th17-cell induction by control and RA-DCs. We utilized also Arg1(-/-) DCs prepared from the BM cells obtained from 12-13 day-old Arg1(-/-) pups but these BM-DCs derived from young pups were ineffective in inducing Th17 cells and therefore did not yield useful information regarding the role of Arg1 in regulation of Th17 cells (Supporting Information Fig. 6B).

DC-derived RA is required for Arg1 expression by DCs

A major source of RA would be the DCs themselves because they highly express RALDH2 in an induced manner by RA as shown in Figure 1. This raised a question if the RA produced by DCs is a significant inducer of Arg1 expression in the cells. We validated this possibility utilizing a RALDH inhibitor (DEAB) and retinol (the substrate for RA). In contrast to control RA-DCs, DEAB-treated RA-DCs were not able to express Arg1 (Fig. 7). Moreover, expression of Arg1 mRNA in the presence of retinol was almost completely suppressed by DEAB (Fig. 7). Thus, the RA, produced in DCs from retinol, is an effective inducer of Arg1 expression.

Discussion

The results of this study implicate RA in regulation of Arg1 expression in the immune system. We identified several 5 RAR binding sites in the *Arg1* gene, which regulate Arg1 expression in response to RA. Arg1, produced by dendritic cells, is involved in regulating naïve T-cell differentiation into FoxP3⁺ Treg cells. In addition, we determined that the RA produced by DCs themselves is a potent inducer of Arg1 expression in DCs.

Most of the studies on expression and function of Arg1 in the immune system has been focused on macrophages and myeloid-derived suppressor cells (MDSC). It has been documented that BM-DCs can express Arg1 in response to Th2/Treg cytokines such as IL-4, IL-13, and IL-10 [7]. It has been reported that DCs, conditioned with tumor cell-derived factors, expressed Arg1 or had increased arginase activity [39, 40]. Recently, RA has been studied extensively for its regulatory function in the immune system [28]. RA can act on naïve T cells during antigen priming and steer their differentiation into FoxP3⁺ Treg cells. Our results show that DCs, generated in the presence of RA, are highly efficient in production of Arg1.

It is well accepted that Arg1 expressed by antigen presenting cells and myeloid cells have immune regulatory functions [13, 41]. A main function of myeloid Arg1 is to deplete arginine and, therefore, to regulate nitric oxide output in myeloid cells co-expressing NOS2 and Arg1 [12]. Depletion of arginine leads to inhibition of T cell responses as exemplified in a cancer model and a *Schistosoma mansoni* infection model [42, 43]. It was also observed that expression of FoxP3 was increased while that of IL-17 was decreased at the mRNA level in the intestine of Arg1(-/-) bone marrow chimeras during acute schistosomiasis [44]. The finding that Treg-cell differentiation is promoted by RA via Arg1 is consistent with the idea that suboptimal activation of T cells through depletion of arginine promotes Tregs. We expected increased generation of Th17 cells with inhibition of arginase, but the inhibition led to somewhat dampened induction of Th17 cells. This result cannot be explained simply by the arginine depletion theory and prompts more research in the future. RA and RA-induced Arg1 may regulate T cell differentiation also through mechanisms other than T cell hypoactivation. RA can affect antigen presenting cells such as dendritic cells and make them into regulatory antigen presenting cells that promote the generation of tolerant T cells [25, 26]. Our results indicate that RA-induced Arg1 would account, in part, for the immune regulatory activity of DCs generated in the presence of RA. One should note that there would also be Arg1-independent effects of RA on Treg differentiation.

The small intestine is a tissue where nutrients such as arginine and retinol are absorbed. Therefore, high levels of RA (an inducer of Arg1) and arginine (the substrate of Arg1) are thought to be present in the small intestinal tissue. This tissue condition would enhance the expression and activity of Arg1. In this regard, we found that antigen presenting cells of the myeloid lineage in the small intestine highly express Arg1. Another function of RA in DCs revealed in this study is to up-regulate SLC7A2 (CAT2B), a major transporter for arginine [29]. Through up-regulation of both Arg1 and CAT2B, RA can make DCs that can effectively catabolize arginine.

Cytokines that are known to regulate Arg1 expression in myeloid cells include IL-4, IL-6, IL-10, IL-13, GM-CSF, TGF- β , and G-CSF, which work, in part, through transcription factors such as STAT6, CAAT/enhancer binding protein, and STAT3 [8, 9, 34, 45]. Also, it has been reported that Liver X Receptor and subsequent activation of PU.1 and IRF 8 are involved in expression of Arg1 in macrophages associated with atherosclerosis [46]. Our results identify a major non-cytokine factor that regulates Arg1 expression in DCs. We found that several RAR binding elements in the 5' upstream regulatory region of the *Arg1* gene are important for the RA-dependent expression of Arg1. Moreover, RA can work together with the Th2 cytokine IL-4 in up-regulation of Arg1 expression. The results indicate also that RA is required for effective up-regulation of Arg1 in developing myeloid cells in response to dendritic cell-inducing cytokines such as GM-CSF and FLT3L. An observation made in this study was that RA does not efficiently induce Arg1 expression in M-CSF-activated myeloid cells. However, small intestinal macrophages highly express Arg1 as demonstrated in this study. This suggests that M-CSF-activated myeloid cells in vitro are quite different from the small intestinal macrophages in arginine catabolism.

Our results indicate that RA induces both RALDH2 and Arg1 in DCs, and the RALDH activity of DCs is required for optimal expression of Arg1 in DCs. These results imply the presence of possible intracellular, autocrine and/or paracrine networks of cell activation by RA for Arg1 expression. Arg1 is another tool of mucosal dendritic cells for regulation of adaptive immune responses in a manner regulated by RA availability.

Materials and Methods

Animals

All the experiments with animals in this study were approved by the Purdue Animal Care and Use Committee. BALB/c mice were purchased from Harlan (Indianapolis, IN). DO11.10 Rag2 (-/-) mice and C57BL/6 mice were purchased from Taconic (Germantown, NY). Arg1-deficient mice (B6.129-Arg1tm1Rki/J) were purchased from the Jackson laboratory and homozygote mice were sacrificed to obtain marrow cells when they were ~ 12 days old because these mice die between 12-14 days after birth [2].

Cell culture

BM-DCs were prepared from femurs by flushing cells with ice-cold PBS. After red blood cell lysis, marrow cells were cultured for 9-10 days at 3×10^6 cells/well in 10 cm plates in complete RPMI 1640 medium (10% FBS) supplemented with mouse GM-CSF (20 ng/ml; PeproTech, Rocky Hill, NJ) or mouse FLT3L (FLT3 ligand; 80-100 ng/ml; eBiosciences). Fifty percent of the medium was replaced with fresh medium containing GM-CSF every 3 days. RA (all-trans retinoic acid; 10 nM; Sigma Aldrich) or Ro41-5253 (a RAR antagonist; 100 nM; Enzo Lifescience) was added to the culture from the beginning. For generation of macrophages, bone marrow cells were cultured in the presence of M-CSF (10 ng/ml; PeproTech) for 7 days with one time replacement of the medium with fresh medium containing M-CSF on day 4. Lipopolysaccharide (LPS; 1 μ g/ml; Sigma Aldrich, St Louis,

MO) was used to activate BM-DCs and macrophages for 24 hours. When indicated, 4-Diethylaminobenzaldehyde (DEAB) was used at the final concentration of 100 μ M.

Microarray and qRT-PCR

Microarray analysis was performed as described previously [47]. Mouse 430 2.0 chips (Affymetrix, Inc.) were used to determine the global gene expression programs of control, RA and Ro41-DC derived from BALB/C or C57BL/6 mice. Raw intensity values were obtained (GCOS, Affymetrix, Inc.) and normalized with the expression values of a housekeeping gene (β -actin). The multiplot module of the GenePattern genomic analysis platform (<http://www.broad.mit.edu/cancer/software/genepattern>) was used to identify genes up- or down-regulated. The raw and processed array data have been deposited at: <http://www.ncbi.nlm.nih.gov/geo> (GSE34324).

qRT-PCR was performed as described previously [47]. The primers used were: CAA GGT GAT GGA AGA GAC CTT (Arg1-F) and TAA GGT AGT CAG TCC CTG GCT T (Arg1-R); TCC AGA AGG TGA TGG AAC AGA (Arg2-F) and CGT GTG GTG AAC TAG GAG TAG (Arg2-R); TCT GCG TTG CCG TCA TTC TG (CAT2B-F) and ATG AAG TAT GAA AGG TAA GCT GA (CAT2B-R); CCA AGC TGG AGT GCG GAG GC (RALDH2-F) and GAT CTT CAC CGT CAC GGT CTT TAC (RALDH2-R); ACC GAC TTG GTC TTTG CCT TC (mRAR -F) and TTC TCC AGC ATT TCC TGG ATC A (mRAR -R); CAC CAT GAC TTT CTC TGA TGG (mRAR -F) and TAC ACG TTC GGC ACC TTT CG (mRAR -R); GGA GAC CGA ATG GAC CTG GA (mRAR -F) and TGG AGC TTC GTC CTC ACT GG (mRAR -R).

Measurements of arginase activity

To measure arginase activity or urea concentration, a commercially available arginase assay kit (BioAssay System, Hayward, CA) was used according to the manufacturer's instruction. BM-DCs (10^5) were harvested and lysed in 100 μ L of 10 mM Tris-HCl (pH 7.4), containing pepstatin A (1 μ M), leupeptin (1 μ M), and Triton X-100 (0.4%). The lysates were centrifuged at 20,000g for 10 minutes at 4°C, and the culture supernatant was examined for arginase activity. The arginase activity is expressed as “nmol/min/ 10^5 cells.”

Bioinformatics and chromatin immunoprecipitation (ChIP) assay

Potential transcriptional binding sites on the *Arg1* gene were identified by TESS program (<http://www.cbil.upenn.edu/cgi-bin/teess/teess>). The chromatin immunoprecipitation assay was performed using the ChIP assay kit according to the manufacture's protocol (Millipore, Temecula, CA). In brief, cultured BM-DCs (5×10^6) were fixed in formaldehyde (1%) and made into cell lysates. Chromosomal DNA-protein complexes were sonicated to generate DNA fragments with their size ranging from 200 to 1000 bp. DNA-protein complexes were immunoprecipitated with rabbit polyclonal antibody to hRAR (Santa Cruz Biotech, Santa Cruz, CA) and protein G-sepharose. The DNA was eluted and analyzed by PCR using the following primer pairs: 5'-GTA ACT GGG AGA ATA CTT GGT T-3' and 5'-TGC CGC GAT TCT AAT CTA CAC-3' (Site A); 5'-AGA GCA GAA GGC TTT GTC AGC-3' and 5'-GAC CAT CCA GAA CTC AGT GCA-3' (Site B); 5'-CTG TGC CAG ATT GAG TGA GAC-3' and 5'-AGC GAC CTC CTG GAA TCC AC-3' (Site C); 5'-AGT GCA GGT AGG ACC ACC AC-3' and 5'-TCA GAA GGA TCT TGT CTC CAG-3' (Site D). Real-time PCR analysis was performed with a 7500 Sequence Detection System using SYBR green Master Mix (Applied Biosystems).

Measurement of the Arg1 promoter activity using a luciferase reporter

The luciferase assay was performed according to the manufacturer's protocol (Luciferase assay system, Promega Corporation, Madison, WI). The 4.8-kb Arg1 promoter described previously [34] was cloned into luciferase reporter vector pGL4.10 (Promega Corporation, Madison, WI). Null mutations were introduced into the putative RAR binding elements in the Arg1 promoter with a mutagenesis kit (Agilent Technology, Santa Clara, CA). Sequencing was performed to verify the desired mutations. BM-DCs (4×10^6) were electro-transfected with the reporter plasmid (15 μ g/transfection) and a reference Renilla luciferase plasmid (pRL, Promega; 1.5 μ g/transfection) using the BioRad Gene Pulser II at 310 V and 1070 μ F (pulsed 2 times) in OPTI-MEM (Life Technologies, Grand Island, NY). The transfected cells were cultured for 90 min and then treated with RA (100 nM) and/or IL-4 (5-10 ng/ml) for 3.5 hours. Luciferase activity was measured with a Synergy HT Multi-Mode Microplate Reader (Bio-Tek).

Isolation and co-culture of CD4⁺ T cells and DCs

CD4⁺ T cells were isolated from the spleen or mesenteric lymph nodes (MLNs) with the CD4 T-cell isolation kit (Miltenyi Biotec, Auburn, CA) as previously described [18]. Cells expressing CD8, CD19, CD25, CD44 and CD69 were further depleted to obtain naïve CD4⁺ T cells. To isolate intestinal DCs, C57BL/6 mice were injected with 2×10^6 B16-FLT3L at the flank [33]. The small intestine was processed as described previously to isolate lamina propria cells [47]. DCs were positively selected with anti-CD11c-microbeads (Miltenyi Biotec).

Naïve CD4⁺ T cells (1×10^5 /well) and DCs (1×10^4 /well) were co-cultured in the presence of staphylococcal enterotoxin B (2 μ g/ml) or OVA₃₂₃₋₃₃₉ peptide (for T cells from DO11.10 Rag2^{-/-} mice) for 6 days in U-bottomed 96-well plates. Recombinant human TGF- β 1 (1 ng/ml) was used for induction of Treg cells. For induction of Th17 cells, TGF- β 1 (5 ng/ml), mIL-6 (20 ng/ml), mIL-21 (10 ng/ml), mIL-23 (10 ng/ml), mIL-1 (10 ng/ml), mTNF- α (10 ng/ml), anti mIL-4 (11B11, 10 μ g/ml), anti-mIFN- γ (XMG2.4, 10 μ g/ml), and LPS (1 μ g/ml) were added. (S)-(2-Boronoethyl)-L-cysteine (BEC, 10-100 μ M; Cayman Chemical, Ann Arbor, MI) and N^ε-hydroxy-nor-L-arginine (Nor-NOHA, 10 μ M; EMD Chemicals, Gibbstown, NJ) were used as arginase inhibitors.

Flow cytometry

T helper cell lineages were determined by intracellular staining for FoxP3, IFN- γ and/or IL-17A. For detection of effector T cells, cells were stained for surface expression of CD4 (RM4-5), CD44 (IM7), CD62L (MEL-14), and/or CD103 (2E7), and activated with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (1 μ M) in the presence of monensin (10 μ g/ml). The cells were fixed and permeabilized for staining with antibodies to IL-17 (TC11-18H10.1) and/or IFN- γ (XMG1.2). Stained cells were analyzed using a BD Canto II (BD Biosciences). For detection of Tregs, cells were stained first with antibodies to surface antigens and then fixed and permeabilized for final staining with anti-FoxP3 (FJK-16s). For staining intracellular Arg1, BM-DCs or intestinal lamina propria cells were fixed in 4% paraformaldehyde, permeabilized, and then stained with a monoclonal antibody for Arg1 (clone 53-207; BD Biosciences) and FITC-goat anti mouse IgG (clone Poly4053; BioLegend, Inc). The cells were incubated with mouse serum and then further stained with indicated antibodies for surface antigens: CD11b (clone M1/70), CD11c (N418), F4/80 (CI:A3-1), and CD103 (2E7).

Western blotting

BM-DCs (4×10^6 cells) were lysed in RIPA Lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) supplemented with a cocktail of protease inhibitors (4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatinA, E-64, bestatin, leupeptin, and aprotinin; from Sigma Aldrich). Lysates (20 μ g of protein/well) were separated by electrophoresis on SDS-polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane (GE Healthcare, Pittsburgh, PA). After blocking overnight in 5% non-fat dried milk at 4°C, the membranes were incubated for 2 hours with a monoclonal antibody to Arg1 (clone 19, BD transduction laboratories) or α -actin (Cell Signaling Technology, Boston, MA). The membranes were further incubated with horseradish peroxidase-conjugated anti mouse IgG (Santa Cruz Biotechnology) or anti-rabbit IgG (Cell signaling Technology, Boston, MA) for 1 h and developed with the ECL detection system (GE Healthcare).

Statistical analysis

Student's paired/unpaired t test was used to determine significant differences between two groups. p values ≤ 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

RA	all-trans retinoic acid
Ro41	Ro41-5253
Arg1	arginase I
BM-DCs	bone marrow-derived dendritic cells
Tregs	FoxP3 ⁺ T regulatory cells
RAR	retinoic acid receptor
RARE	retinoic acid response element
BEC	(S)-(2-Boronoethyl)-L-cysteine
Nor-NOHA	N -hydroxy-nor-L-arginine
ChIP	chromatin immunoprecipitation

Fig. 1. Retinoic acid induces Arg1 expression in BM-DCs

(A) A genome-wide microarray multi-plot. BM-DCs were prepared by culturing mouse bone marrow cells with GM-CSF for 9-10 days. RA (10 nM) or Ro41-5253 (Ro41; 100 nM) was added as indicated during this culture. BALB/C BM-DCs were examined in the immature state or after activated with LPS for 24 h. (B) Real-time qRT-PCR analysis of Arg1 mRNA expression in BM-DCs induced with GM-CSF. The data are presented relative to β -actin expression. (C) BM-DC lysates were examined for expression of cellular Arg1 protein expression. Lysates of ~0.3 million DCs were loaded for the western blotting study. β -actin was used as a loading control. (D) Induction of Arg1 mRNA and protein in FLT3L-induced BM-DCs. BM-DCs were prepared by culturing mouse bone marrow cells with FLT3L for 9-10 days. RA or Ro41 was added as indicated during this culture. Real-time qRT-PCR and flow cytometry were performed. (E) Arginase enzyme activity was measured in BM-DC lysates. (F) Expression of Arg2, CAT2B, and RALDH2 mRNA in the DCs populations. Data are shown as mean + SEM of 3-4 data sets (D-F; pooled) or mean + SD of triplicate measurements (B, CAT2B in F; representative) from 3-4 experiments performed. For most experiments except that of panel D, GM-CSF-induced DCs were used. Significant differences between the groups (Student's t test) are indicated as * p <0.05, ** p <0.001 and *** p <0.0001.

Fig. 2. Intestinal DCs express Arg1

(A) Expression of Arg1 protein by spleen versus small intestinal lamina propria DCs. Cell purity is shown before and after isolation. Lysates of ~0.3 million DCs were loaded for the western blotting study. -actin was used as a loading control. Data shown are representative of three experiments performed. (B) Flow cytometric detection of Arg1 expression by CD11c^{high} CD103⁺ DCs, CD11c^{high} CD103⁻ DCs, and CD11b^{high} F4/80⁺ macrophages isolated from the small intestinal lamina propria. The mean fluorescence intensity for the antigen presenting cells in the small intestine and spleen is also shown (right). Data are shown as mean + SEM of results pooled from three experiments. Significant differences between the groups (Student's t test) are indicated as *p<0.05, **p<0.001 and ***p<0.0001.

Fig. 3. RA and IL-4 cooperatively induce Arg1 expression

(A, B) The expression of Arg1 (A) mRNA and (B) protein in DCs induced by RA and IL-4 is shown. (C, D) The expression of Arg1 (C) mRNA and (D) protein in M-CSF-activated marrow cells in response to RA is shown. BM cells were cultured for 9-10 days with GM-CSF in the presence of RA (10 nM), Ro41 (100 nM), and/or IL-4 (5 ng/ml). BM-derived macrophages were prepared by culturing marrow cells with M-CSF for 7 days. Real time PCR and western blotting were performed. Data are shown as mean + SD of triplicate measurements (A, C) and are from one experiment representative of three performed (B, D). Significant differences between the groups (Student's t test) are indicated as * $p < 0.05$, ** $p < 0.001$ and *** $p < 0.0001$.

Fig. 4. RAR binding elements in the 5' upstream region of the *Arg1* gene mediate the induction of *Arg1* expression in response to RA

(A) The expression of mRNA for *RAR* α , *RAR* β and *RAR* γ in BM-DCs developed with RA or Ro41 is shown. (B) The *Arg1* promoter is activated in response to retinoic acid. The luciferase reporter was electro-transfected into control BM-DCs and luciferase activity was measured following activation of the transfected cells with indicated factors (RA and/or IL-4) for 3.5 hours. (C) Presence of putative RAREs in the *Arg1* promoter is shown. (D) Chromatin immunoprecipitation assay for RAR binding to the *Arg1* promoter. (E) The *Arg1* promoter construct with mutations in the three RARE sites is defective in driving the transcription. Pooled data obtained from 4-5 experiments are shown with SEM (A, B, E) or differences between duplicated measurements are shown (D). All experiments were performed at least 3 times. Significant differences between the groups (Student's t test) are indicated as * $p < 0.05$, ** $p < 0.001$ and *** $p < 0.0001$.

Fig. 5. Arg1, expressed by dendritic cells in response to RA, promotes the generation of FoxP3⁺ T cells

(A) RA-treated DCs are efficient in induction of Treg cells. A co-culture method used to assess the activity of DCs in induction of Treg cells is shown in the diagram. (B) Treg-cell induction was determined after the exposure of RA-DCs to Arg1 inhibitors at 10 μ M. Data are shown as mean + SEM of results pooled from 3-6 experiments. (C and D) Cell-free Arg1 from RA-DCs can promote Treg-cell induction. Conditioned media of control, RA and Ro-DCs (10^6 cells/ml for 48 h) were obtained from the cells and examined for (C) Arg1 protein and (D) effects on Treg-cell induction. For culture with T cells, the conditioned media were obtained by culturing 8-day old control, RA and Ro-DCs for additional 2 days in the absence of RA or Ro41. Splenocytes enriched with naïve CD4⁺ T cells isolated from DO11.10 rag (-/-) mice were cultured in the conditioned medium (70% conditioned medium and 30% fresh medium) in the presence of OVA₃₂₃₋₃₃₉ (1 μ g/ml) and BEC (100 μ M). Five to six days later, frequencies of CD4⁺FoxP3⁺ T cells were determined. Data are shown as mean + SEM of results pooled from three experiments. Significant differences between the groups (Student's t test) are indicated as * p <0.05, ** p <0.001 and *** p <0.0001.

Fig. 6. Arg1-deficient dendritic cells are inefficient in the induction of Treg cells

(A) Arg1 protein expression was determined for wild type and Arg1-deficient BM-DCs. β -actin was used as a loading control. (B) Comparison of the activities of wild type and Arg1-deficient BM-DCs in the induction of Treg cells. Control, RA and Ro41-DCs were generated with GM-CSF from the marrow cells of WT and Arg1-deficient mice. Ten day-old BM-DCs were examined for Arg1 protein expression or Treg-cell-inducing activity. Data are shown as mean + SEM of results pooled from three experiments. *p=0.0078, Student's t test.

Fig. 7. DC-derived RA induces Arg1 expression

8 day-old RA-DCs were cultured in the presence of DEAB (100 μ M), retinol (100 nM) or DEAB plus retinol for 3 days. qRT-PCR was performed to determine Arg1 mRNA expression. Data are shown as mean + SD of triplicate measurements and are from one experiment representative of four performed. Significant differences between the groups (Student's t test) are indicated as * $p < 0.05$ and ** $p < 0.001$.