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IL-4 and Retinoic Acid Synergistically Induce Regulatory Dendritic Cells Expressing Aldh1a2

Bing Zhu,* Thomas Buttrick,* Ribal Bassil,* Chen Zhu,* Marta Olah,* Chuan Wu,* Sheng Xiao,* William Orent,* Wassim Elyaman,* and Samia J. Khoury*†

Although activated inflammatory monocytes (IMCs) and inflammatory dendritic cells (IDCs) are potent T cell suppressors, non-activated IMCs and IDCs promote T cell activation and Th1/Th17 cell differentiation. In this study, we investigated how to reduce the proinflammatory properties of IMCs and IDCs and further convert them into immune regulatory dendritic cells (DCs). We found that IL-4 and retinoic acid (RA) cotreatment of GM-CSF–differentiated IDCs synergistically induced the expression of aldehyde dehydrogenase family 1, subfamily A2, a rate-limiting enzyme for RA synthesis in DCs. IL-4 plus RA–treated IDCs upregulated CD103 expression and markedly reduced the production of proinflammatory cytokines upon activation. IL-4 plus RA–treated IDCs strongly induced CD4+Foxp3+ regulatory T cell differentiation and suppressed Th1 and Th17 differentiation. Mechanistically, the transcription factors Stat6 and RA receptor β play important roles in aldehyde dehydrogenase family 1, subfamily A2, induction. In addition, IL-4 and RA signaling pathways interact closely to enhance the regulatory function of treated DCs. Adoptive transfer of IL-4 plus RA–treated DCs significantly increased regulatory T cell frequency in vivo. Direct treatment with IL-4 and RA also markedly suppressed actively induced experimental autoimmune encephalomyelitis. Our data demonstrate the synergistic effect of IL-4 and RA in inducing a regulatory phenotype in IDCs, providing a potential treatment strategy for autoimmune diseases. The Journal of Immunology, 2013, 191: 3139–3151.

*Center for Neurologic Diseases, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115; and †Abou Haidar Neuroscience Institute, American University of Beirut, Beirut 1007 2020, Lebanon

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Address correspondence and reprint requests to Dr. Samia J. Khoury and Dr. Bing Zhu, Harvard Medical School, Room 641, New Research Building, 77 Avenue Louis Pasteur, Boston, MA 02115. E-mail addresses: skhoury@rics.bwh.harvard.edu (S.J.K.) and bing6335@yahoo.com (B.Z.)

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; Aldh1a2, aldehyde dehydrogenase family 1, subfamily A2; BMDC, bone marrow–derived dendritic cell; ChIP, chromatin immunoprecipitation; Ct, threshold cycle; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; IDC, inflammatory DC; IMC, inflammatory monocyte; L-NIL, N6-(1-iminoethyl)-L-lysine; MOG, myelin oligodendrocyte glycoprotein; nNOS, neural nitric oxide synthase; nTreg, naturally occurring regulatory T cell; PT, pertussis toxin; RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; Treg, regulatory T cell.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/$16.00. We therefore explored how to reduce the proinflammatory properties of IMCs and IDCs and further convert them into immune regulatory dendritic cells (DCs).

Regulatory or tolerogenic DCs function to delete or suppress pathogenic T cells (10, 11). A major focus of research has been to generate regulatory DCs that induce Ag-specific Foxp3+ regulatory T cells (Tregs) for the prevention and treatment of autoimmune diseases, graft-versus-host disease, and rejection in transplantation (12, 13). In the steady state, CD103+ DCs isolated from the intestinal lamina propria and mesenteric lymph nodes efficiently induce Tregs through the production of retinoic acid (RA) and TGF-β (14, 15). These DCs have enriched mRNA and protein expression of aldehyde dehydrogenase family 1, subfamily A2 (Aldh1a2), a rate-limiting enzyme for RA synthesis in DCs (14, 16). In addition, CD103+ DCs play an important role in oral tolerance (17). However, intestinal inflammation may reduce Aldh1a2 and TGF-β expression in CD103+ DCs, and impair their ability to induce Tregs, but favor the emergence of IFN-γ–producing T cells (18). In addition, there is significant loss of CD103+ DCs during colonic inflammation (19).

RA has been shown to induce Treg differentiation in the presence of TGF-β and to reciprocally suppress Th17 differentiation (20–24). RA also sustains the stability of naturally occurring Tregs (nTregs) in the presence of IL-6 (25). Mechanistically, RA was shown to enhance TGF-β–induced Smad3 expression and phosphorylation and to reduce the expression of IL-6Ra, IFN regulatory factor-4, and IL-23R in T cells (26). In vivo, RA treatment suppresses Th1- and Th17-mediated immunopathology in various autoimmune disease models, including experimental autoimmune encephalomyelitis (EAE) (27–29), uveoretinitis (30), myositis (31), colitis (32–34), rheumatoid arthritis (35), and type 1 diabetes (36, 37).

Vitamin A (retinol) is converted by alcohol dehydrogenase into retinal, which is further converted by Aldh1a2 into RA in DCs (38). It has been reported that Aldh1a2 mRNA expression in GM-CSF–
cultured splenic or bone marrow–derived DCs (BMDCs) could be enhanced by ~5- to 10-fold with IL-4 or RA treatment (39, 40). Because DCs are present in inflammatory sites at high frequencies, and are different from resident DCs (41), we explored how to convert proinflammatory DCs into regulatory DCs through the induction of Aldh1a2. Although separate treatment with IL-4 or RA only weakly induced Aldh1a2 mRNA and enzyme activity in DCs, we found that a combination of IL-4 and RA treatment synergistically increased Aldh1a2 mRNA expression by ~300-fold and strongly induced its protein expression as well as enzyme activity. IL-4 plus RA treatment also markedly inhibited production of proinflammatory cytokines in DCs. We further characterized the signaling events, revealing the close interaction between IL-4 and RA signaling pathways. In addition, strong regulation by IL-4 plus RA-treated DCs, as well as by direct IL-4 plus RA treatment in vivo suggests potential clinical implications for autoimmune diseases.

Materials and Methods

Animals and reagents
Female C57BL/6 mice and Stat6–/– mice (B6 background) were obtained from The Jackson Laboratory. The 2D2 myelin oligodendrocyte glycoprotein (MOG) TCR transgenic mice and Foxp3-GFP knock-in mice were provided by V.K. Kuchroo (Harvard Medical School, Boston, MA), and were then crossed to generate 2D2 Foxp3-GFP mice. Animals were used at 6–10 wk of age. All animal procedures performed in this study were approved by the Institutional Animal Care and Use Committee of Harvard Medical School. All-trans retinoic acid, retinol, and retininal were obtained from Sigma-Aldrich. LE135, N6-(1-iminoethyl)-L-lysine (L-NIL), and LE135, N6-(1-iminoethyl)-L-lysine (L-NIL), and NAD were purchased from Cayman Chemicals. Recombinant cytokines were obtained from R&D Systems. FACS Abs and agonistic anti-CD40 were purchased from BD Biosciences, eBioscience, or BioLegend.

IMC isolation and differentiation
B6 mice were immunized with an emulsion of 100 μl PBS and 100 μl CFA containing 0.5 mg heat-inactivated Mycobacterium tuberculosis (H37Ra; Difco Laboratories). Each animal also received 200 ng pertussis toxin (PT; List Biological Laboratories) i.v. on days 0 and 2 postimmunization. On day 10, splenic Ly-6G− cells were purified by depletion Ly-6G+ cells with anti-Ly-6G biotin (clone IAB) and anti-biotin microbeads (Miltenyi Biotec). CD11b+ cells were then purified by anti-CD11b microbeads (Miltenyi Biotec). After staining with anti-Ly-6C–FITC (clone AL-21), CD11b+Ly-6C–Ly-6G− IMCs were purified by FACS sorting. To differentiate IMCs into IDCs, cells were loaded onto 0.4 mg/ml collagen gel for 10 min at 37°C. CD11c+ IDCs were further purified with CD11c microbeads (Miltenyi Biotec) or FACS sorting.

T cell differentiation
The 2D2 CD4+ T cells were isolated by CD4-positive selection kit, and CD4+CD25hiCD44− naïve T cells were further purified by cell sorting. CD4+ T cells (1 × 10^6 cells/well) were cultured with the same number of IDCs and 20 ng/ml MOG35–55 peptide (neutral Th0 condition). For Th1 differentiation, cells were treated with 20 ng/ml IL-6 and 3 ng/ml TGF-β. For Treg differentiation, cells were treated with 5 ng/ml TGF-β. After 3 d of culture, cells were collected for intracellular cytokine staining and FACS analysis.

Flow cytometry
For surface staining, isolated cells were blocked with 10 μg/ml mouse Fc Block (BD Biosciences) at 4°C for 5 min and labeled with fluorochrome-conjugated Abs and 7-aminoactinomycin D (7-AAD), including isotype controls, for 15 min at 4°C. After 3× washing, cells were analyzed on the FACSCalibur (BD Biosciences). Intracellular cytokine and Foxp3 staining was performed according to the BD Biosciences protocol. Data analysis was performed by FlowJo software and gated on 7-AAD− live cells.

ALDEFLUOR staining
Aldehyde dehydrogenase activity was quantified using ALDEFLUOR kit (Stemcell Technologies). Briefly, 2 × 10^6 cells from each sample were stained with ALDEFLUOR substrate for 30 min at 37°C. Negative control samples were pretreated with ALDH inhibitor diethylaminobenzaldehyde for 15 min before substrate staining. After treatment with mouse Fc Block (BD Biosciences), cells were stained with cell surface markers and 7-AAD, and analyzed by flow cytometry.

Real-time PCR (RT-PCR)
Total RNA was extracted using Absolutely RNA miniprep kit (Agilent Technologies). cDNA was synthesized using SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). mRNA expression of Aldh1a2, Rara, Rarb, Rarg, Il4ra, Il13ra1, Il13ra2, Arg1, Retnla, Chi3L3, and Ctgdh was examined using TaqMan gene expression assay (Applied Biosystems). Triplicate samples were examined in each condition. A comparative threshold cycle (C_T) value was normalized for each sample using the following formula: ΔC_T = C_T (gene of interest) − C_T (GAPDH), and the relative expression was then calculated using the Equation 2−ΔΔC_T.

Immunoblotting
Cells were lysed with radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors. Protein concentrations were quantified with a bicinchoninic acid kit assay (Pierce). A total of 20 μg protein from each sample was loaded into each lane of 10% NuPAGE Bis-Tris gels and then separated by electrophoresis. Proteins were transferred onto a polyvinylidene difluoride membrane. The blot was blocked in StartinBlock buffer (Thermo Scientific) and then incubated overnight with the primary Abs. After washing, the blot was incubated with HRP-labeled goat anti-mouse or anti-rabbit IgG (Sigma-Aldrich), and developed with an Immobilon chemiluminescent HRP substrate (Millipore). To improve staining specificity, RA receptor (RAR)β imunoblotting was carried out using nuclear protein extracts, which were prepared with NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific). Anti-mouse Aldh1a2 (N-20) and anti-RARβ (C-19) Abs were obtained from Santa Cruz Biotechnology. Anti-Stat6, anti-phospho-Stat6 ( Tyr445), and anti-histone H3 Abs were obtained from Cell Signaling Technology. Anti–β-actin Ab was obtained from Sigma-Aldrich.

Chromatin immunoprecipitation
Forty-million BMDCs were treated with IL-4 and RA for 3, 6, 9, and 12 h. Chromatin immunoprecipitation (ChIP) assays were carried out using SimpleChIP enzymatic chromatin immunoprecipitation kit (Cell Signaling). Briefly, cells were fixed with 1% formaldehyde and then digested with micrococcal nuclease to induce optimal genomic DNA fragmentation. After sonication, samples were subjected to immunoprecipitation with anti-Stat6 (Cell Signaling; D3H4), anti-RARβ (Santa Cruz; C-19), or control rabbit IgG. DNA samples were then purified, and SYBR Green RT-PCR was performed to quantify the amount of specific DNA sequences. Genomic DNA sequences 1 kb upstream of the transcription start site of Aldh1a2, Il4ra, and Rarb genes were obtained from Genome Browser (University of California, Santa Cruz, Santa Cruz, CA), and primers were designed at site 1 (−1 ~ −500 bp) and site 2 (between −500 ~ −1000 bp) using PrimerQuest (Integrated DNA Technologies). Primer sequences for Aldh1a2 promoter are as follows: site 1, 5′-GGCCGTATGCAAGTGCTTCCTC-3′ (forward) and 5′-TGCGTGTATATGGCGAGGTGTCACCAG-3′.
Alcohol dehydrogenase gene expression in IDCs. CD11b^Ly-6C^high IMCs were isolated from CBA/PT-immunized mice and differentiated into IDCs with GM-CSF treatment for 48 h (8). IDCs were then treated with IL-4 and RA either separately or in combination for 24 h. RT-PCR shows that whereas separate treatment with IL-4 or RA significantly induced Aldh1a2 gene expression, combined IL-4 plus RA treatment had a much stronger synergistic effect (Fig. 1A). Titration of different doses of IL-4 and RA showed that Aldh1a2 was optimally induced at 20 ng/ml IL-4 in combination of 1 μM RA (Supplemental Fig. 1A, 1B). Aldh1a2 gene expression increased almost linearly during the first 24 h of treatment and then reached a plateau (Supplemental Fig. 1C). In addition, Aldh1a2 protein expression was only detectable in IL-4 plus RA-treated IDCs (Fig. 1B), along with markedly increased enzyme activity of aldehyde dehydrogenase (Fig. 1C).

We asked whether other Th2 cytokines and RA precursors would function similarly as IL-4 and RA. We found that IL-13 and RA also synergistically induced Aldh1a2, whereas IL-5 did not, consistent with the almost undetectable Ili1a expression in IDCs (Supplemental Fig. 1D, 1E). The weaker effect of IL-13 compared with IL-4 also correlated with significantly lower expression of Il13ra1 than Ili1a (Supplemental Fig. 1D, 1E). Furthermore, RA precursors retinol and retinal synergistically induced Aldh1a2 expression when combined with IL-4, and there was no significant difference in effectiveness among RA, retinol, and retinal (Supplemental Fig. 1F).

To test the stability of Aldh1a2 expression, IDCs were first treated with IL-4 and RA for 24 h. After several washes to remove IL-4 and RA, we activated these IDCs with IFN-γ and agonistic anti-CD40 treatment for another 24 h in the presence of GM-CSF. Aldh1a2 expression was not significantly altered by activation (Fig. 1D). In contrast, the production of IL-6, IL-12p70, and TNF-α from IL-4 plus RA–treated IDCs was significantly reduced, suggesting that IL-4 plus RA treatment strongly suppressed the proinflammatory properties of IDCs (Fig. 1E).

We examined IDC phenotype after 24 h of IL-4 and/or RA treatment. IL-4 treatment increased CD11c expression, but RA mildly reduced CD11c expression. IL-4 plus RA–treated IDCs retained a high level of CD11c expression that is comparable to IL-4–treated IDCs (Fig. 1F). Interestingly, whereas separate IL-4 or RA treatment did not induce CD103 expression, ~70% of IL-4 plus RA–treated IDCs were positive for CD103 (Fig. 1F). In addition, a majority of IL-4 plus RA–treated IDCs expressed MHC class II, CD80, PD-L1, and PD-L2 (Supplemental Fig. 2). CD86 expression was increased in IL-4 plus RA–treated IDCs. B7-H3 and B7-H4 were expressed on very few cells across groups. These data suggest that IL-4 plus RA–treated IDCs express CD11c and molecules critical for Ag presentation and specifically upregulated CD103 expression.

T cell regulation by IL-4 plus RA–treated IDCs

To examine whether Aldh1a2 expression in IDCs regulates T cell function, we cultured various IDCs with CD4^+ T cells from 2D2 MOG TCR transgenic mice, in the presence of 20 μg/ml MOG35–55 peptide. Whereas RA-treated IDCs had reduced ability to induce T cell proliferation, IL-4 and IL-4 plus RA–treated IDCs induced similar levels of T cell proliferation as control IDCs (Fig. 2A). However, T cells cultured with IL-4 plus RA–treated IDCs had a dramatically reduced production of IFN-γ and IL-17, whereas IL-2 production was unchanged (Fig. 2B). We further examined the function of various IDCs in T cell differentiation. Under neutral (Th0) condition, both IL-4–treated and RA-treated IDCs reduced the frequency of IFN-γ– and IL-17–producing T cells, but IL-4 plus RA–treated IDCs had the strongest effect (Fig. 2C). Under
Th17-polarizing condition, IL-4 plus RA–treated IDCs decreased IL-17–producing T cells by ~90% (Fig. 2C). The inhibition of Th17 differentiation with IL-4 plus RA–treated IDCs was fully reversed by the treatment with LE135 (an RAR antagonist), but not with L-NIL (a NO synthase 2 inhibitor) or nor-NOHA (an arginase inhibitor) (Fig. 2D), suggesting that reduction in Th17 differentiation was dependent on RA production from IL-4 plus RA–treated IDCs. When IL-4 plus RA–treated IDCs were cultured together with 2D2 T cells and control IDCs, Th17 polarization was also reduced by ~90%, and this reduction was again fully reversed.

FIGURE 1. IL-4 and RA cotreatment synergistically induces Aldh1a2 expression in IDCs. (A) IDCs were treated with IL-4 (20 ng/ml) and/or RA (1 μM) for 24 h, and Aldh1a2 mRNA was quantified by RT-PCR. (B) Aldh1a2 protein expression was examined by immunoblotting in nontreated and IL-4 and/or RA–treated IDCs at 24 h. (C) Aldehyde dehydrogenase activity was examined by ALDEFLUOR staining in IDCs treated for 24 h. Data quantifying the median fluorescence intensity are shown in the right graph. (D) IDCs were treated with IL-4 and RA for 24 h. After washing, cells were either nonactivated or activated with IFN-γ (20 ng/ml) and agonistic anti-CD40 (20 μg/ml) for 24 h. Aldh1a2 expression was quantified by RT-PCR. (E) Culture supernatants collected from experiments described in (D) were analyzed by Milliplex assay for cytokine production. (F) Phenotype of IDCs was analyzed by flow cytometry after treatment for 24 h. Expression of CD11c and CD103 is shown in solid lines, and isotype control staining is shown in tinted gray areas. Labels over individual columns indicate the statistical significance compared with the first column on left (nontreated group). Labels on the connecting lines show the statistical significance among other columns. Data are representative of two to three independent experiments. *p < 0.05, #p < 0.01. n.s., Not significant.
FIGURE 2. IL-4 plus RA–treated IDCs inhibit T cell production of IFN-γ and IL-17. (A) CD4+ T cells from 2D2 MOG TCR transgenic mice were cultured with nontreated IDCs or IDCs treated with IL-4, RA, or IL-4 plus RA in the presence of MOG35–55 peptide (20 μg/ml). T cell proliferation assay was performed after 48 h. (B) IL-2, IFN-γ, and IL-17 concentrations in the culture supernatants of above experiments were measured. (C) Naive 2D2 CD4+ T cells were cultured with various IDCs in neutral (Th0) or Th17-polarizing condition for 72 h. Intracellular staining for IFN-γ and IL-17 was examined in gated CD4+ T cells. (D) Intracellular staining of 2D2 CD4+ T cells after cultured with control IDCs and/or IL-4 plus RA–treated IDCs in Th17-polarizing condition. LE135 (RAR antagonist), L-NIL (NO synthase 2 inhibitor), and nor-NOHA (arginase I inhibitor) were used at 1 μM, 1 mM, and 0.5 mM, respectively. In these experiments, T cells were used at 1 × 10^5/well, and IDCs were used at 5 × 10^4/well in a round-bottom 96-well plate. Data are representative of three independent experiments. *p < 0.05, #p < 0.01. n.s., Not significant.
by LE135 treatment, suggesting that RA production from IL-4 plus RA–treated IDCs dominantly suppresses Th17 differentiation induced by control IDCs (Fig. 2D). FACS analysis of 7-AAD staining shows that coculture with IL-4 plus RA–treated IDCs did not increase T cell death (data not shown), consistent with the observed normal T cell proliferation (Fig. 2A).

Under Treg-polarizing condition, IL-4 plus RA–treated IDCs consistently showed the strongest effect in inducing CD4+Foxp3+ T cells, which was largely reversed by LE135 treatment (Fig. 3A). This suggests that RA production from IL-4 plus RA–treated IDCs plays a critical role in enhanced Treg induction. Interestingly, IL-4 plus RA–treated IDCs induced more Foxp3+ T cells than IL-17–producing T cells under the Th17 condition (Fig. 3B), suggesting that IL-4 plus RA–treated IDCs can block IL-6 signaling in T cells. Because increased Treg differentiation could be due to enhanced conversion from non-Treg cells and/or enhanced expansion of nTregs, we tested these possibilities in our model. CD4+Foxp3− non-Treg cells were purified from 2D2 Foxp3KI mice and cocultured with various IDCs in Treg-polarizing condition (Fig. 3C). The data show that IL-4 plus RA–treated IDCs most efficiently increased conversion from non-Treg cells to Treg cells. To test the function of IL-4 plus RA–treated IDCs on Treg expansion, we purified Foxp3+ nTregs from 2D2 Foxp3KI mice and cultured them with control IDCs or IL-4 plus RA–treated IDCs in the presence of MOG35–55 peptide and TGF-β for 48 h (Fig. 3D). IL-4 plus RA–treated IDCs induced much stronger expansion of nTregs than control IDCs, and IDCs themselves did not proliferate.

**Signaling mechanism for Aldh1a2 induction in IDCs**

Because Stat6 activation is a major pathway downstream of IL-4 and IL-13 signaling (45), we investigated the role of Stat6 in IL-4 plus RA–treated IDCs in Aldh1a2 induction. Immunoblotting shows that from 30 min to 2 h after IL-4 and RA treatment, there was strong Stat6 phosphorylation on tyrosine 641, whereas the total amount of Stat6 remained unchanged (Fig. 4A). Using IDCs from Stat6 knockout mice, we show that Aldh1a2 expression was abrogated in IL-4–treated IDCs, not affected in RA-treated IDCs, and reduced to a level similar to RA-treated IDCs in IL-4 plus RA–treated IDCs (Fig. 4B). These data suggest that Stat6 is necessary in IL-4–induced Aldh1a2 expression, but not in RA-induced Aldh1a2 expression.

To test whether Stat6 directly binds to the promoter region of Aldh1a2, we performed chromatin immunoprecipitation (ChIP) PCR assays. BMDCs were used due to the insufficient cell num-

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**FIGURE 3.** IL-4 plus RA–treated IDCs promote Treg differentiation through RA production. (A) CD4+ T cells from 2D2 Foxp3 knock-in mice were cultured with various IDCs in Treg-polarizing condition for 72 h. Foxp3+GFP+ cell frequency was examined in gated CD4+ T cells by flow cytometry. (B) Frequencies of Foxp3+GFP+ and IL-17–producing CD4+ T cells were determined after coculturing with control or IL-4 plus RA–treated IDCs in Th17-polarizing condition. (C) CD4+Foxp3− cells were isolated from 2D2 Foxp3 knock-in mice and cultured with various IDCs under Treg-polarizing condition. Efficiency of conversion from Foxp3− to Foxp3+ T cells was examined. (D) CD4+Foxp3+ nTregs were isolated from 2D2 Foxp3 knock-in mice, and cultured with control or IL-4 plus RA–treated IDCs under Treg-polarizing condition. Proliferation assay was performed after 48 h. In these experiments, T cells were used at 1 × 10^5/well, and IDCs were used at 5 × 10^4/well in a round-bottom 96-well plate. Data are representative of three independent experiments. *p < 0.01.
mers of IDCs, and we found that IL-4 plus RA treatment also synergistically induced Aldh1a2 expression in BMDCs (Supplemental Fig. 3A). ChIP PCR shows that Stat6 directly binds to the promoter region of Aldh1a2 in wild-type BMDCs (Fig. 4C), whereas there was no binding in Stat6−/− BMDCs (Fig. 4D). Additional negative control PCR assays were performed with primers specific for a chromosome DNA region not associated with transcription factor binding (42). The amplification was the same between control Ig and anti-Stat6 immunoprecipitated DNA from Stat6+/+ BMDCs (data not shown), suggesting that the changes in Aldh1a2 ChIP PCR are specific.

All-trans RA specifically binds to RAR, which form heterodimers with retinoid X receptor (RXR) to regulate gene transcription (38, 46). We examined the expression of RAR subtypes in IDCs using RT-PCR (Fig. 5A). Whereas IL-4 plus RA treatment synergistically induced Rarb expression, Rara and Rarg expression was significantly reduced (Fig. 5A). A time course study shows that Rarb induction mostly occurred within the first 3 h of IL-4 and RA treatment (Fig. 5B). To examine RARβ protein expression, we harvested nuclear protein extracts to enhance the Ab specificity in immunoblotting. The data show that IL-4 plus RA–treated IDCs expressed the highest levels of RARB (Fig. 5C). Because IL-4 plus RA treatment also synergistically induced Rarb expression in BMDCs (Supplemental Fig. 3B), we used these cells in RARB ChIP PCR assays. The data show that RARB binds to the promoter regions of both Rarb and Aldh1a2 (Fig. 5D, 5E), suggesting that RARB not only functions as positive feedback in inducing its own expression, but also directly drives Aldh1a2 expression. Negative control PCR again shows the same amplification for control Ig and anti-RARB immunoprecipitated DNA samples (data not shown).

In addition, we found that IL-4 and RA signaling pathways closely interact and mutually enhance each other (Fig. 6). Synergistic induction of Rarb by IL-4 and RA treatment was lost in Stat6−/− IDCs (Fig. 6A). ChIP PCR shows that Stat6 binds to Rarb promoter in IL-4 plus RA–treated Stat6+/+ BMDCs (Fig. 6B), whereas no binding was seen in Stat6−/− BMDCs (Fig. 6C). Furthermore, RA treatment was found to induce Il4ra gene expression (Fig. 6D). In support of this, ChIP PCR data show that RARB binds to the Il4ra promoter (Fig. 6E). Thus, IL-4 enhances RA signaling by inducing RARB expression, and RA enhances IL-4 signaling by inducing Il4ra expression.

To directly test the role of Stat6 and RARB in Aldh1a2 expression, we performed a luciferase assay (Fig. 7). RAW264.7 cells were transfected with plasmid vectors expressing Stat6, RARB, or both. A significant increase in Aldh1a2 promoter activity was detected only in the cells overexpressing both Stat6 and RARB (Fig. 7A). In addition, when Stat6 and RARB–transfected RAW264.7 cells were activated by IL-4 and RA treatment, more robust activation of the Aldh1a2 promoter was observed (Fig. 7B), suggesting that activation of transcription factors Stat6 and RARB by IL-4 and RA treatment further enhances the Aldh1a2 promoter activity.

In vivo IL-4 and RA treatment strategies

To study the function of IL-4 plus RA–treated DCs in vivo, we treated BMDCs with IL-4 and RA for 48 h, and then pulsed them with MOG35–55 peptide for 4 h before transferring to naive 2D2 mice at 10^7 cells/mouse on days 0 and 4. On day 7, significantly increased frequencies of CD4+Foxp3+ Treg cells were observed in the spleen and blood of IL-4 plus RA–treated DC recipients, but not in control DC recipients (Fig. 8A). The absolute numbers of CD4+ Foxp3+ Treg cells were also significantly increased in the spleens of IL-4 plus RA–treated DC recipients (3.81 ± 0.13 million in IL-4 plus RA–treated DC recipients versus 1.43 ± 0.41 million in nontreated mice and 1.44 ± 0.1 million in control DC recipients, p < 0.05). Whereas transfer of control or IL-4 plus RA–treated DCs induced few IL-17–producing CD4 T cells in the spleen, IFN-γ–producing T cells were mildly induced in both groups, with no significant difference between them (Fig. 8B).

Both IL-4 and RA treatments have been reported to be protective in EAE (27, 29, 47). Based on our results in IDCs, we asked
whether IL-4 and RA cotreatment might offer even better EAE protection. We treated MOG35–55-immunized mice with IL-4, RA, or IL-4 plus RA from day 0 to day 15 after immunization (Fig. 9). Mean maximal disease score was significantly reduced in the IL-4 and RA treatment groups, and it was further significantly reduced in IL-4 plus RA–treated group (Fig. 9A, Supplemental Table I). Histology on spinal cord tissues harvested on day 20 shows that cotreated mice had the lowest number of inflammatory foci and the lowest percentage of white matter area with myelin damage (Fig. 9B). Splenocytes were harvested on day 20 and stimulated with 20 μg/ml MOG35–55 for 48 h, and the cytokine production was examined by Luminex assay (Fig. 9C). IL-17 was significantly reduced in all three treated groups, but IFN-γ, as well as IL-6 and GM-CSF (data not shown) were only significantly reduced in IL-4 plus RA–treated group. The production of these proinflammatory cytokines was significantly lower in IL-4 plus RA–treated group than in separately treated groups. In addition, splenic CD11b+Ly-6Chigh cells isolated from IL-4 plus RA–treated group on day 20 had the strongest increase in the expression of Aldh1a2 and Rarb (Fig. 9D). However, Foxp3+ Treg frequency in the spleen was not significantly different among these groups on day 9 postimmunization, probably due to the polyclonal T cell repertoire in these mice or due to migration of Ag-specific Tregs to the immunization sites or CNS (data not shown).

In summary (Fig. 10), our data show that IL-4 and RA treatment synergistically induces the expression of Aldh1a2 in IDCs, and treated IDCs promote Treg differentiation while suppressing Th1 and Th17 cell differentiation through induced RA production. Mechanistically, Stat6 and RARβ are important transcription factors in driving Aldh1a2 expression. IL-4 and RA signaling pathways interact closely, with Stat6 inducing Rarb expression and RARβ inducing Il4ra expression. RARβ also positively induces its own expression. In vivo, adoptive transfer of IL-4 plus RA–treated IDCs increased Treg frequency in an Ag-specific fashion, and direct treatment with IL-4 and RA also suppressed actively induced EAE.

**Discussion**

We and others have reported that IMCs are present at high frequencies in inflammatory tissues and readily differentiate into IDCs upon GM-CSF signaling (4, 8, 9). Nonactivated IDCs efficiently present Ags to T cells and promote Th1/Th17 cell differentiation (8, 9). In contrast, CD103+ DCs in the gut express Aldh1a2 and produce RA to regulate T cell differentiation (14, 15). They play a critical role in oral tolerance by inducing Tregs (48). In this study, we discovered the synergistic role of IL-4 and RA treatment on the induction of Aldh1a2 expression in IDCs at the mRNA, protein, and enzyme activity levels. Yokota et al. (40) reported that GM-CSF–differentiated BMDCs and splenic DCs had increased Aldh1a2 expression compared with Flt-3 ligand–differentiated
DCs, and IL-4 treatment further increased Aldh1a2 mRNA expression by ~6- to 7-folds. Similar levels of Aldh1a2 induction were reported in IL-4–treated mesenteric lymph node DCs (49). In addition, RA treatment has also been shown to increase Aldh1a2 mRNA expression by 5- to 10-folds in BMDCs (39). In IDCs, we found that Aldh1a2 mRNA was weakly induced by separate IL-4 or RA treatment, but the protein expression or enzymatic activity of aldehyde dehydrogenase was not induced. In contrast, the combined IL-4 and RA treatment in IDCs increased Aldh1a2 mRNA expression by ~300-folds, and markedly induced Aldh1a2 protein expression and enzymatic activity.

Our data suggest that Stat6 is necessary for IL-4–induced Aldh1a2 expression. It is well known that Stat6 is a major IL-4 signaling molecule and may bind to specific DNA motifs in the promoters of target genes (45). Bioinformatics analysis with Explain 3.0 (Biobase) suggests that multiple Stat6 binding sites may exist within 1 kb upstream of Aldh1a2 transcription start site (data not shown). ChIP PCR clearly shows the binding of Stat6 to Aldh1a2 promoter region 3 h after treatment. Interestingly, no positive binding was detected when ChIP PCR assay was performed at 6, 9, and 12 h. It is possible that 3 h posttreatment is the most active time point when phosphorylated Stat6 binds to the Aldh1a2 promoter. Another possibility is that the formation of a more complex transcription activation machinery at later time points may prevent the Ab from recognizing the specific epitope on Stat6. However, these negative data serve as additional specificity controls, in addition to the results from Stat6−/− control and negative control PCR.

All-trans RA binds to RARα, RARβ, and RARγ, which form heterodimers with RXRα, RXRβ, and RXRγ (46). RA binding induces a conformational change of the heterodimers, resulting in the dissociation of corepressor complexes and the recruitment of coactivator complexes, and subsequent changes in target gene expression (46). We found that Rarb mRNA expression was synergistically induced by IL-4 and RA treatment, in parallel with the induction of Aldh1a2. Rarb induction reached peak level at 3 h after treatment, earlier than the peak Aldh1a2 induction (24 h). It has been reported that Rarb promoter contains a RA response element, which may bind RARβ (50, 51). In tumor cells, RA treatment induces the expression of RARβ, which functions as a tumor suppressor gene (52). Our ChIP analysis showed that RARβ was able to bind directly to its own gene promoter, providing further evidence for the positive feedback gene induction. Also using ChIP PCR, RARβ was shown to bind to the promoter.

**FIGURE 6.** Interaction of IL-4 and RA signaling pathways. (A) IDCs were derived from wild-type and Stat6−/− mice, and treated with IL-4, RA, or IL-4 plus RA for 24 h. Rarb expression was compared with nontreated IDCs by RT-PCR. (B) BMDCs from wild-type mice were treated with IL-4 plus RA for 3 h. ChIP PCR was performed with control IgG and anti-Stat6 Ab, and PCR primers were specific for the two sites within 1 kb upstream of Rarb. (C) ChIP experiment was the same as in (B), with BMDCs derived from Stat6−/− mice. (D) IDCs from wild-type mice were treated with RA for 24 h. Il4ra expression was compared with nontreated IDCs by RT-PCR. (E) BMDCs from wild-type mice were treated with IL-4 plus RA for 3 h. ChIP PCR was performed with control IgG and anti-RARβ Ab, and PCR primers were specific for two sites within 1 kb upstream of Il4ra. Data are representative of two to three independent experiments. *p < 0.05, †p < 0.01. n.s., Not significant.

**FIGURE 7.** Role of Stat6 and RARβ in activating Aldh1a2 promoter. (A) RAW264.7 cells were transfected with Stat6 and/or Rarb expression plasmids, Aldh1a2 promoter reporter plasmid, and Renilla luciferase plasmid. Aldh1a2 promoter activity was examined by luciferase assay after 48 h. (B) The same luciferase assay was performed as above, and RAW264.7 cells were treated with IL-4 plus RA after plasmid transfection. Data are representative of three independent experiments. *p < 0.01. n.s., Not significant.
region of Aldh1a2, explaining the synergistic effect with IL-4–induced Stat6 signaling. In support of this, analysis with Explain 3.0 software shows two potential RAR binding sites within 1 kb upstream of Aldh1a2 transcription start site (data not shown). Luciferase assay further confirms the synergistic effects of Stat6 and RARβ in the activation of Aldh1a2 promoter. RARβ is known to play a unique role in RA treatment for cancer and skin diseases (53) and in RA-induced neuronal differentiation (54). However, our data do not exclude the possible role of RARα and RARγ in Aldh1a2 induction. The relative contribution of these RAR subtypes will need to be studied in specific strains of gene knockout mice.

Colonic inflammation was shown to reduce Aldh1a2 expression in CD103+ DCs (18). Therefore, the stability of Aldh1a2 expression appears to be a critical factor in generating functional immunoregulatory DCs. We found that the expression of Aldh1a2 was sustained in IL-4 plus RA–treated DCs even after strong activation with a combination of IFN-γ and agonistic anti-CD40 for 24 h. In addition, the production of proinflammatory cytokines such as IL-6, IL-12p70, and TNF-α was markedly reduced in IL-4 plus RA–treated DCs after activation. We found that IL-4 and RA treatment in DCs also synergistically induced expression of important markers for M2 cells, such as arginase I, Ym-1, resistin-like α, and insulin-like growth factor (data not shown), suggesting that IL-4 plus RA treatment could be an effective approach to polarize type 2 myeloid cells in general (55, 56). Although characterization of the mechanisms behind these findings is out of the scope of this study, we found that IL-4 and RA signaling pathways closely interact. IL-4 synergistically induced Rarb expression with RA, and RA treatment induced the expression of Il4ra expression. Close interactions between two signaling pathways may help explain the strong effect of IL-4 and RA treatment in inducing the alternative activation of DCs and the strong and stable induction of Aldh1a2 expression.

Along with the strong induction of Aldh1a2, IL-4 plus RA–treated DCs have a significantly enhanced ability in promoting Treg and suppressing Th1/Th17 differentiation, and this was dependent on RA production. Even under strong Th17-polarizing condition, IL-4 plus RA–treated DCs still induced significantly more Treg cells than IL-17–producing T cells. Furthermore, IL-4 plus RA–treated DCs dominantly suppressed Th17 differentiation induced by control DCs. Although similar effects were observed with direct RA treatment during T cell differentiation (20–24), our

FIGURE 8. Transfer of IL-4 plus RA–treated BMDCs increases Treg frequency in vivo. (A) Control DCs and IL-4 plus RA–treated DCs were pulsed with MOG35–55 peptide (20 μg/ml) for 4 h and transferred into naive 2D2 mice at 10^7 cell/mouse on days 0 and 4. On day 7, splenocytes and blood leukocytes were harvested, and Foxp3+ cell frequency in CD4+ T cells was determined by flow cytometry. Quantification data are shown in graphs on the right. (B) IFN-γ and IL-17 production from splenic CD4+ T cells was examined by intracellular cytokine staining and flow cytometry. Quantification data are shown in the graph on the right. Data are representative of two independent experiments with three mice per group in each experiment. *p < 0.05, **p < 0.01. n.s., Not significant.
data demonstrate the role of IL-4 plus RA treatment in converting otherwise proinflammatory DCs to immune regulatory DCs. In this study, we show that combination treatment with IL-4 and RA induced CD103 expression on the majority of IDCs, which share the strong Aldh1a2 expression as the gut CD103+ DCs. Although RA and GM-CSF have been suggested to induce CD103 expression on GALT DCs, our data suggest that a Th2 cytokine environment may synergistically induce the expression of CD103 as well as Aldh1a2. In contrast to the typical tolerogenic DCs that are phenotypically immature (57), IL-4 plus RA–treated IDCs express high levels of CD11c, MHC-II, and other costimulatory molecules. This is consistent with their efficient Ag-presenting function in vitro. In addition, in vivo IL-4 plus RA treatment in EAE model did not suppress MOG35–55-induced T cell proliferation in the recall (data not shown).

RA treatment in vivo has shown strong effects in suppressing autoimmune diseases in various animal models (27–37). An alternative strategy is to induce RA production from endogenous cells, especially in cells actively migrating to the inflammatory sites, such as IMCs and IDCs. We show in this study that after pulsing with MOG35–55, IL-4 plus RA–treated BMDCs significantly increased Treg frequency when adoptively transferred into MOG TCR transgenic mice. Due to the cell number limitation, we were not able to observe consistent EAE suppression by adoptive transfer of IL-4 plus RA–treated DCs, which may need to be performed at multiple time points during EAE priming. Although we focused on DCs in this study, it is possible that IL-4 plus RA treatment may induce Aldh1a2 expression in other cells as well, such as macrophages or mesenchymal stem cells, which are being considered for cell therapy in autoimmune diseases (58, 59).

Our data show that direct treatment of IL-4 and RA significantly suppressed actively induced EAE disease. This is manifested by reduced CNS inflammation and reduced production of a number of proinflammatory cytokines in the recall assay. In addition, Aldh1a2 and Rarb mRNA expression in the splenic Ly-6Chigh myeloid cells was significantly increased. In addition to their effects on IMCs and IDCs, IL-4 plus RA treatment may directly target T cells in vivo, as well as modulating the immune function of resident DCs, macrophages, and microglia. Therefore, the combined IL-4 and RA treatment may represent a novel treatment strategy for Th1- and Th17-mediated autoimmune diseases. Recently, IL-15 was shown to convert RA from an immunoregulatory to a proinflammatory function on DCs (60). It remains to examine whether IL-4 cotreatment could reverse such an effect. In summary, our findings could have important clinical implications in treating autoimmune diseases and inducing immune tolerance in transplantation settings.

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Disclosures
The authors have no financial conflicts of interest.

FIGURE 9. Cotreatment with IL-4 and RA effectively suppresses EAE. (A) B6 mice were immunized with MOG35–55 on day 0 to induce EAE, and received daily treatment from day 0 to day 15. Five groups were set up, as follows: nontreated, vehicle treated, IL-4 treated, RA treated, and IL-4 plus RA treated. Data were pooled from two independent experiments showing similar results. (B) Spinal cord tissues were harvested on day 20 from three mice per group, and processed for H&E and Luxol fast blue staining. The number of inflammatory foci and the percentage of white matter area with demyelination were quantified. (C) Splenocytes were harvested on day 20 from three mice per group, and activated with 20 μg/ml MOG35–55 for 48 h in vitro. Cytokine production in the supernatant was examined with Milliplex assays. (D) Splenic CD11b+Ly-6Chigh cells were isolated on day 20, and mRNAs of Aldh1a2 and Rarb were quantified by RT-PCR. *p < 0.05, #p < 0.01.

FIGURE 10. Illustration of IL-4 and RA signaling pathways leading to Aldh1a2 induction. Increased RA production from IDCs promotes Treg differentiation and suppresses Th1 and Th17 differentiation.


Supplementary Figure 1. Aldh1a2 expression induced by Th2 cytokines, RA, and RA precursors. A) Different IL-4 concentrations were tested in the presence of 1 μM RA for Aldh1a2 expression. B) Dose response of RA was tested in the presence of 20 ng/ml of IL-4 on Aldh1a2 expression. C) Time course of Aldh1a2 expression after 20 ng/ml IL-4 and 1 μM RA co-treatment. D) IDCs were treated with IL-4, IL-13 and IL-5, with or without RA for 24 h. Aldh1a2 expression was examined with RT-PCR. E) Il4ra, Il13ra1 and Il5ra expression was examined in untreated IDCs by RT-PCR. F) IDCs were treated with retinol, retinal or RA (all at 1 μM), with or without IL-4 (20 ng/ml) for 24 h. Aldh1a2 expression was examined with RT-PCR. *, P < 0.05; #, P < 0.01; n.s., not significant. Data are representative of two independent experiments.

Supplementary Figure 2. Expression of MHC class II, CD80, CD86, PD-L1, PD-L2, B7-H3, B7-H4 on IDCs after 24 h of treatment. Specific staining is shown in solid lines, and isotype control staining is shown in tinted gray areas.

Supplementary Figure 3. IL-4 and RA treatment induces Aldh1a2 and Rarb expression in BMDCs. BMDCs were left untreated or treated with IL-4 and/or RA for 24 h, and the expression of Aldh1a2 (A) and Rarb (B) was determined by RT-PCR. #, P < 0.01.
**Supplemental Table I: IL-4 and RA treatment suppresses EAE**

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of animals</th>
<th>EAE onset (days post-immunization)</th>
<th>Mean maximal disease score</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-treated</td>
<td>8</td>
<td>9.8 ± 0.6</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>vehicle-treated</td>
<td>8</td>
<td>11.4 ± 0.6</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>IL-4-treated</td>
<td>9</td>
<td>15.3 ± 1.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.9 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RA-treated</td>
<td>9</td>
<td>14.6 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.7 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-4+RA-treated</td>
<td>8</td>
<td>16.3 ± 1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.6 ± 0.3&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
</tbody>
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<sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01, <sup>c</sup>P<0.001 when comparing each treated group with non-treated group in one-way ANOVA test followed by Bonferroni post tests.

<sup>d</sup>P < 0.01 when comparing IL-4+RA-treated group with IL-4 or RA-treated group in one-way ANOVA test followed by Bonferroni post tests.
Supplementary Fig. 1