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*J Immunol* 2012; 189:2697-2701; Prepublished online 15 August 2012; doi: 10.4049/jimmunol.1201248
http://www.jimmunol.org/content/189/6/2697

Supplementary Material  http://www.jimmunol.org/content/suppl/2012/08/15/jimmunol.1201248.DC1
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Cutting Edge: 4-1BB Controls Regulatory Activity in Dendritic Cells through Promoting Optimal Expression of Retinal Dehydrogenase

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Dendritic cells (DC) in the gut promote immune tolerance by expressing retinal dehydrogenase (RALDH), an enzyme that promotes retinoic acid, which aids differentiation of Foxp3+ inducible regulatory T cells (iTreg) in the intestinal mucosa. How RALDH expression is regulated is unclear. We found that 4-1BB (CD137), a member of the TNFR family, together with CD103, marked mesenteric lymph node DC with the highest level of RALDH activity, and ligation of 4-1BB maintained RALDH expression in these gut DC. Moreover, 4-1BB signals synergized with those through TLR2 or GM-CSFR to promote RALDH activity in undifferentiated DC. Correspondingly, 4-1BB–deficient mice were impaired in their ability to generate iTreg in the GALT when exposed to oral Ag, and 4-1BB–deficient mesenteric lymph node DC displayed weak RALDH activity and were poor at promoting iTreg development. Thus, our data demonstrate a novel activity of 4-1BB in controlling RALDH expression and the regulatory activity of DC.

The Journal of Immunology, 2012, 189: 2697–2701.

The mucosa of the gut is the largest surface in the body and is continuously exposed to exogenous Ags that may be innocuous (food, commensal bacteria) or pathogenic (invasive bacteria, food allergens) (1, 2). As such, lymphocytes distributed throughout the GALT must remain immunologically hyporesponsive to many Ags. Migratory intestinal dendritic cells (DC), marked by CD103 expression, control immune homeostasis in the GALT through the promotion of high frequencies of Foxp3+ regulatory T cells (Treg) that suppress Th1 and Th17 populations in effector sites, such as the lamina propria (3, 4). This regulatory DC activity is specifically associated with expression of retinal dehydrogenases (RALDH or aldehyde dehydrogenase) that direct retinoic acid (RA) production (5, 6). RA is made by DC in both Peyer’s patches (PP) and mesenteric lymph nodes (MLN) and can direct T cells to have gut-homing potential by inducing α4β7 and CCR9 (7, 8). Furthermore, RA synergizes with TGF-β in promoting the differentiation of Foxp3+ inducible Treg (iTreg) while suppressing Th17 development (9–11). Although the RA-producing GALT DC have been well characterized in terms of activity, the factors that control the capacity of these DC to express RALDH and, therefore, exhibit suppressive function, are not clear.

4-1BB (CD137, TNFRSF9) (12) is a receptor in the TNFR superfamily that is mostly known as a co-signaling molecule promoting the activation and expansion of peripheral effector T cells (13, 14). Although the majority of research on 4-1BB has been directed to T cells, it is becoming clear that this molecule can influence other lineages of cells in both positive and negative manners, as exemplified by our recent data showing that 4-1BB expressed on bone marrow progenitors limits myelopoiesis and DC development (15). In further pursuing this latter observation, we found unusual constitutive expression of 4-1BB on MLN DC that overlapped to a great extent with CD103. We now show that these 4-1BB+ gut DC expressed the highest levels of RALDH. 4-1BB was also induced on undifferentiated DC through TLR2 and GM-CSFR, molecules that have been implicated in driving the development of RALDH+ regulatory DC. Moreover, signaling through 4-1BB promoted optimal RALDH in these DC and imparted regulatory activity in the cells. Correlating with these observations, Foxp3+ iTreg conversion to oral Ag was dampened in the GALT of 4-1BB–deficient mice, and MLN DC from 4-1BB–deficient mice displayed an attenuated ability to promote iTreg differentiation as the result of reduced expression of RALDH. These data define a new and novel role for 4-1BB in DC biology.

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Received for publication June 5, 2012. Accepted for publication July 16, 2012.

This work was supported by National Institutes of Health Grants AI42944 and AI85291 (to M.C.) and Grant AI050265 (to H.C.). This is manuscript no. 1149 from the La Jolla Institute for Allergy and Immunology.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; iTreg, inducible regulatory T cell; MDC, mesenteric lymph node dendritic cell; MFI, mean fluorescence intensity; MLN, mesenteric lymph node; PP, Peyer’s patches; RA, retinoic acid; RALDH, retinal dehydrogenase; SDC, spleen dendritic cell; Treg, regulatory T cell; wt, wild-type.

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Materials and Methods

**Mice**

4-1BB–deficient (16) and OT-II mice on a BL/6 background were bred at La Jolla Institute for Allergy and Immunology. C57BL/6J (CD45.2) and C57BL/6J (CD45.1) mice were purchased from The Jackson Laboratory. All experiments were in compliance with the regulations of the La Jolla Institute for Allergy and Immunology animal care committee in accordance with guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care.

**Cells**

DC from spleens and mesenteric lymph nodes (SDC and MDC, respectively) were isolated by MACS with CD11c MicroBeads, as described previously (15). Naïve OT-II CD4+ T cells were further purified by cell gating by setting CD4+CD25+CD45RBo sperm cells as CD4+CD25+CD45RBhi, which results in negligible contamination of Foxp3+ cells, as described previously (17). Lymphocytes were isolated from spleens, lymph nodes, and PP by treatment with collagenase D (Roche), followed by mashing through a 70-μm cell strainer. Intraepithelial lymphocytes and lamina propria lymphocytes were isolated as described (11).

**RALDH induction in SDC**

A total of 2 × 10^5 SDC was cultured with a cytokine, GM-CSF (10–1000 pg/ml), or TLR2 ligands zymosan (0.25–25 μg/ml; Sigma-Aldrich) and Pam-3-cys (0.1–10 μg/ml; InvivoGen) for 48 h. Where indicated, rat IgG (KLH/G1-2-2; 25 μg/ml), anti-4-1BB (3H3; 25 μg/ml), and inhibitors of signaling pathways were added at the start of culture. For blockade of signaling, the following were used: ERK inhibitor U0126 (5 μM), PI3K inhibitor LY294002 (5 μM), NF-κB inhibitor Bay 11-7082 (1 μM, all from Sigma-Aldrich), and Wnt/β-catenin inhibitor XAV939 (5 μM; sc-296704; Santa Cruz Biotechnology). The activity of RALDH was determined by ALDEFLUOR staining, as described (18).

**In vitro lineage differentiation of CD4+ T cells**

A total of 1 × 10^5 naïve OT-II T cells and 2 × 10^5 DC (MDC or SDC) was cultured in the presence of OVA323-339 peptide (0.1–1 μM) for 4 d. In some cultures, SDC were preactivated with zymosan (2.5 μg/ml) together with IgG or anti-4-1BB (25 μg/ml) for 48 h, washed three times with PBS, and then cocultured with OT-II cells. Where indicated, exogenous cytokines and reagents were added at the start of culture. Th cell lineages were determined by intracellular staining for IFN-γ (Th1) and Foxp3 (Treg) after restimulating cells with PMA and ionomycin (Sigma-Aldrich) for 5 h in the presence of GolgiPlug (BD).

**Oral tolerance and in vivo conversion of Treg**

We exploited an oral tolerance model to observe the conversion of naïve OT-II cells into Foxp3+ Treg in vivo. Briefly, wild-type wt or 4-1BB−/− mice (CD45.2) received naïve OT-II cells (1 × 10^5, CD45.1) and 1 d later were challenged with OVA (1%; Sigma-Aldrich) in the drinking water for 5 d. Water containing OVA was changed approximately every 36 h. The conversion of Treg was analyzed by intracellular staining of Foxp3+ Treg from donor (CD45.1) and recipient (CD45.2) CD4+ T cells.

**Statistics**

All statistical analyses were done with a two-tailed Student t test.

Results and Discussion

**4-1BB is expressed on CD103+ regulatory MDC**

Although SDC do not constitutively express 4-1BB, we found that approximately half of MDC expressed 4-1BB when analyzed immediately ex vivo (Fig. 1A). Moreover, the 4-1BB+ DC population overlapped with the CD103+ population to a great extent, implying that 4-1BB expression might correlate with RALDH activity and production of RA that were reported to associate with CD103 expression (Fig. 1B, top left panel). To assess this, we used a sensitive flow technique with a fluorescent substrate for aldehyde dehydrogenase (ALDEFLUOR). Fluorescence strongly correlates with both the level of mRNA for Aldh1a2 (RALDH2) that is expressed in DC and the ability of DC to promote generation of Foxp3+ iTreg (18, 19).

Most interestingly, the subset of MDC positive for both CD103+ and 4-1BB displayed a higher level of activity of RALDH than did CD103+ cells that were negative for 4-1BB (Fig. 1B, top right panel; CD103+4-1BB− versus CD103+4-1BB+, 57.1 versus 38.9% ALDEFLUOR+, and 19.128 versus 11.441 mean fluorescence intensity [MFI] of ALDEFLUOR). To control for RALDH activity, the assay was performed in the presence of diethylaminobenzaldehyde, a RALDH inhibitor (data not shown). When CD103+ MDC were further subdivided based on the levels of CD103 and 4-1BB, the activity of RALDH was found to be markedly stronger in 4-1BB high or low cells compared with 4-1BB negative cells (Fig. 1B, bottom panel; quadrant gates 1–4; CD103+4-1BB− versus CD103+4-1BB+, 46.2 versus 71.9% ALDEFLUOR+, and 12,944 versus 28,097 MFI of ALDEFLUOR; CD103+4-1BB− versus CD103+4-1BB+, 35.7 versus 45.1% ALDEFLUOR+ and 10,804 versus 12,222 MFI of ALDEFLUOR). Similarly, gating only on the level of expression of 4-1BB revealed MDC with progressively increasing RALDH activity (Fig. 1C; 4-1BB− versus 4-1BBlow versus 4-1BBhi, 20.2 versus 46.8 versus 62.6% ALDEFLUOR+ and 6,339 versus 15,265 versus 20,879 MFI of ALDEFLUOR). Thus, 4-1BB is a marker of RALDH activity in migratory GALT DC.

**4-1BB signaling maintains and augments RALDH**

To assess whether 4-1BB signaling might control RALDH expression or activity, MDC were cultured in vitro in the absence of stimulation or were stimulated with an agonist Ab to 4-1BB. Although MDC strongly lost RALDH activity over a 48-h period without further stimulation, triggering 4-1BB allowed a large proportion of the cells to maintain high levels of active RALDH (Fig. 2A).

Regulatory GALT DC are thought to develop or mature in the periphery from RALDH+ precursors in response to GM-CSFR and/or TLR2 signals (18, 20). To potentially mimic
this process and assess whether 4-1BB might participate in such a differentiation event, we cultured SDC that do not constitutively express RALDH or 4-1BB with GM-CSF or zymosan (25 μg/ml). Activity of RALDH was assayed by ALDEFLUOR staining. Percentages of ALDEFLUOR+ cells are indicated. (B–F) MACS-isolated SDC were stimulated with GM-CSF and zymosan for 48 h. Where indicated, agonist anti–4-1BB (25 μg/ml) or rat IgG was added at the start of culture. (B) 4-1BB expression in SDC stimulated with GM-CSF (100 pg/ml) and zymosan (25 μg/ml). (C) Activity of RALDH in SDC was determined by ALDEFLUOR staining when treated with GM-CSF (10 pg/ml), zymosan (2.5 μg/ml), and Pam-3-cys (1 μg/ml) for 48 h. (D and E) SDC, pretreated with zymosan in the presence of IgG or agonist anti–4-1BB for 48 h, were cultured with naïve OT-II CD4 T cells and OVA peptide (0.01 μg/ml). Results are representative of three experiments. **p > 0.01.

4-1BB is a signaling receptor for RALDH induction in DC. (A) MDC, isolated by MACS, were cultured for 1 or 2 d in the absence of stimulation with control IgG or stimulated with an agonist Ab to 4-1BB (25 μg/ml). Activity of RALDH was assayed by ALDEFLUOR staining. Where indicated, TGF-β (0.01 μg/ml) cultured with naive OT-II T cells and OVA peptide (0.01 μg/ml) were restimulated with IgG or agonist anti-4-1BB for 48 h, were added at the start of culture when SDC were stimulated with zymosan (25 μg/ml). ERK, U0126 (5 μM) for 4 d. Where indicated, TGF-β (5 ng/ml) and LE540, a pan antagonist of the RA receptor (2), were added. Treg/Th1 development was determined by intracellular staining after restimulation of cells with PMA and ionomycin. Representative flow plot (D) and the percentage of Foxp3+ T cells in individual cultures (E) are shown. (F) Inhibitors for signaling pathways were added at the start of culture when SDC were stimulated with zymosan (25 μg/ml). ERK, U0126 (5 μM); PI3K, Ly (5 μM); NF-kB (1 μM); Wnt/β-catenin (5 μM). Results are representative of three experiments. **p > 0.01.

Defective Foxp3 iTreg generation to oral Ag in 4-1BB–deficient mice

The expression of RALDH in GALT DC is thought to be crucial for inducing tolerance against orally encountered Ags by promoting RA production that contributes to the development of Foxp3+ iTreg (5, 6). We then performed oral-tolerance experiments in 4-1BB–deficient mice. To assess induction of Foxp3+ Treg and focus on a potential role of 4-1BB on APC populations, we adoptively transferred naive (Foxp3− CD25−) OVA-specific OT-II T cells (CD45.1+) into 4-1BB−/− mice and fed the animals OVA in their drinking water. When transferred into wt mice, a significant number of Th cells strongly upregulated Foxp3 in the GALT after oral Ag treatment (Fig. 3). In contrast, Treg induction was dramatically attenuated in the MLN, PP, and lamina propria lymphocytes when T cells were transferred into 4-1BB−/− mice. Defective Treg generation was specific to the GALT because conversion was moderately increased in the spleen of 4-1BB−/− recipients (Fig. 3). This implied that 4-1BB−/− mice possess an environment in the GALT that is less favorable for inducible Treg development and that this is related to attenuated regulatory activity in mucosa-resident APC populations.

4-1BB–deficient MDC express low levels of RALDH and are poor at inducing Treg

Although DC are believed to be the primary inducer of Treg induced by oral Ags in the GALT, some studies suggested that macrophages and intestinal epithelial cells may also contribute to the generation and/or maintenance of these iTreg (21, 22). To investigate whether the defective Treg induction that we observed in 4-1BB–deficient animals was likely related to al-
tered DC activity, we isolated DC from the MLN. This is the main location where naïve CD4 T cells are converted into iTreg with oral Ag (5, 21). Significantly, the percentage of MDC in 4-1BB−/− mice expressing RALDH activity was strongly reduced and the level of activity was much lower than in wt MDC (Fig. 4A, 4B). Moreover, the level of active RALDH in 4-1BB−/− MDC was comparable to that observed in 4-1BB+ MDC from wt mice (compare with data in Fig. 1). In contrast, SDC from wt or 4-1BB−/− mice were indistinguishable and essentially negative for RALDH (Fig. 4A). The reduced activity of RALDH in 4-1BB−/− MDC was paralleled by a lower level of Aldh1a2 (RALDH2) mRNA (Supplemental Fig. 1A) and was not due to decreased numbers of the CD103+ population (Supplemental Fig. 1B).

MDC from 4-1BB−/− mice did not show any obvious surface phenotypic differences compared with wt MDC, including expression of CD80, CD86, and MHC class II (Supplemental Fig. 1B). 4-1BB ligand was found on wt MDC at low levels and at higher levels on 4-1BB−/− MDC (Supplemental Fig. 1C), corresponding to our previous results that showed that endogenously expressed 4-1BB antagonizes the expression of 4-1BB ligand (15). To assess the functional activity of MDC, we cultured naïve wt OT-II CD4 T cells with cells isolated from wt or 4-1BB−/− mice and determined their relative ability to support Th1 (medium-only culture) or Foxp3 iTreg (TGF-β culture) development driven by OVA peptide. MDC from 4-1BB−/− mice induced more Th1 cells than did MDC from wt mice (Fig. 4C), and most significantly promoted fewer Foxp3+ iTreg (Fig. 4D). In contrast, SDC isolated from 4-1BB−/− mice displayed normal activity for the development of Th1 cells and Treg (Supplemental Fig. 2A, 2B).

The defective iTreg-promoting ability of 4-1BB−/− MDC was strongly recovered when RA was added exogenously into culture, suggesting that it was largely due to the reduced activity of RALDH (Fig. 4E). However, Treg conversion in the presence of RA was moderately lower with 4-1BB−/− MDC than with wt MDC, indicating that other factors might also contribute to the aberrant regulatory activity. Neutralization of IFN-γ or IL-12 strongly enhanced the differentiation of iTreg induced by 4-1BB−/− MDC (Fig. 4F), but no synergy was observed when both were inhibited, implying that IL-12 was aberrantly expressed in these cultures. In accordance with this, more IL-12p70 was produced from 4-1BB−/− MDC when they were stimulated with Cpg DNA (Fig. 4G). Interestingly, IL-12p70 production was augmented when MDC were costimulated with IFN-γ and IL-12 (10 μg/ml) added at the start of culture. MDC from wt and 4-1BB−/− mice were stimulated as indicated for 24 h. IL-12p70 production was measured by ELISA in culture supernatants. Results are representative of one experiment. **p < 0.01, ***p < 0.001.
Disclosures
The authors have no financial conflicts of interest.

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