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Stimulation of Dopamine Receptor D5 Expressed on Dendritic Cells Potentiates Th17-Mediated Immunity

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Dendritic cells (DCs) are responsible for priming T cells and for promoting their differentiation from naive T cells into appropriate effector cells. Emerging evidence suggests that neurotransmitters can modulate T cell-mediated immunity. However, the involvement of specific neurotransmitters or receptors remains poorly understood. In this study, we analyzed the role of dopamine in the regulation of DC function. We found that DCs express dopamine receptors as well as the machinery necessary to synthesize, store, and degrade dopamine. Notably, the expression of D5R decreased upon LPS-induced DC maturation. Deficiency of D5R on the surface of DCs impaired LPS-induced IL-23 and IL-12 production and consequently attenuated the activation and proliferation of Ag-specific $CD4^+$ T cells. To determine the relevance of D5R expressed on DCs in vivo, we studied the role of this receptor in the modulation of a $CD4^+$ T cell-driven autoimmunity model. Importantly, D5R-deficient DCs prophylactically transferred into wild-type recipients were able to reduce the severity of experimental autoimmune encephalomyelitis. Furthermore, mice transferred with D5R-deficient DCs displayed a significant reduction in the percentage of Th17 cells infiltrating the CNS without differences in the percentage of Th1 cells compared with animals transferred with wild-type DCs. Our findings demonstrate that by contributing to $CD4^+$ T cell activation and differentiation to Th17 phenotype, D5R expressed on DCs is able to modulate the development of an autoimmune response in vivo. ***The Journal of Immunology*****, 2012, 188: 3062–3070.**

Dendritic cells (DCs) are the most efficient type of APCs and are specialized in the initiation of immune responses by directing the activation and differentiation of naive T cells (1). In the absence of foreign Ags, immature DCs can present self-antigens on MHC molecules and, by stimulating $CD4^+$ regulatory T cells (Tregs), induce peripheral tolerance (2). In contrast, maturation induced by recognition of pathogen-associated molecular patterns allows DCs to migrate into lymph nodes and promote the differentiation of naive $CD8^+$ T cells and $CD4^+$ T cells into CTLs and the appropriate effector Th subset, respectively (1). Depending on the cytokine milieu, DCs can stimulate the polarization of naive $CD4^+$ T cells into Th1, Th2, and Th17 cells (3, 4). In particular, the release of IL-12 by DCs can induce $CD4^+$ T cells to adopt the IFN-γ–secreting Th1 phenotype. However, the production of IL-6 or IL-23 by DCs can promote $CD4^+$ T cells to acquire the IL-17–producing Th17 fate. Because of the key role of DCs in modulating the interface between immunity and tolerance, their function must be tightly regulated (5). Traditionally, it has been thought that the functions of immune cells such as T cells and DCs are regulated mainly by cytokines. However, a number of more recent studies have shown that immune system cells can also be regulated by neurotransmitters (6–11). Consistent with this, both primary and secondary lymphoid organs are highly innervated by sympathetic ends that store dopamine (DA) (12, 13).

DA is an important neurotransmitter in the CNS and is involved in the control of locomotion, emotion, cognition, and neuroendocrine secretion (14). The first and rate-limiting step in DA biosynthesis is the conversion of L-tyrosine to (*S*)-2-amino-3-(3,4-dihydroxyphenyl)propanoic acid (L-DOPA), which is catalyzed by the enzyme tyrosine hydroxylase (TH). This compound is subsequently metabolized by aromatic amino acid decarboxylase to produce cytosolic DA (15). Cytosolic DA can also be taken up from the extracellular environment through plasma-membrane dopamine transporters (DATs) (16). Once inside the cell, DA can either be inactivated by monoamine oxidases (MAO-A and -B) (17) or can be stored in intracellular vesicles. Storage is mediated by type 1 and type 2 vesicular monoamine transporters (VMATs 1 and 2) that mobilize cytosolic DA toward vesicular stores (18). In some catecholaminergic cells, DA can adopt a third fate and be further processed by dopamine β-hydroxylase (DβH) to yield norepinephrine (19).

DA exerts its effects by stimulating dopamine receptors (DARs) expressed on the cell surface. Five DARs have been identified to date: D1R, D2R, D3R, D4R, and D5R (20, 21). All of these receptors are hepta-spanning membrane proteins that belong to the superfamily of G protein-coupled receptors. Based on their se-

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Abbreviations used in this article: DA, dopamine; DAR, dopamine receptor; DAT, dopamine transporter; DC, dendritic cell; DβH, dopamine β-hydroxylase; D5RKO, D5R-knockout; EAE, experimental autoimmune encephalomyelitis; iDC, immature dendritic cell; MAO, monoamine oxidase; mDC, mature dendritic cell; MFI, mean fluorescence intensity; MS, multiple sclerosis; NET, norepinephrine transporter; pAb, polyclonal Ab; pMOG, myelin oligodendrocyte glycoprotein 35–55 peptide; SCH, SCH23390; SERT, serotonin transporter; SKF, SKF38393; TH, tyrosine hydroxylase; Treg, regulatory T cell; VMAT, vesicular monoamine transporter; WT, wild-type.

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quence homology, signal transduction machinery, and pharmacological properties, DARs have been classified into two subgroups. D1R and D5R are type I DARs, which couple with stimulatory Gα subunits, and D2R, D3R, and D4R are type II DARs, which couple to inhibitory Gα subunits (20).

Much evidence regarding the functional relevance of DAR expression and DA signaling in immune cells has amassed over the years. Some immune cells such as Tregs have been found to contain substantial amounts of DA and other catecholamines. These cells also constitutively express TH, an enzyme necessary to synthesize DA. Effector T cells, in contrast, contain only trace amounts of DA (22, 23). In Tregs, endogenous DA signals through D1R/D5R in a paracrine/autocrine manner resulting in the downregulation of Treg function (23). In DCs, recent pharmacological evidence suggests that the antagonism of D1R/D5R expressed on DCs could impair the polarization of naive $CD4^+$ T cells toward the Th17 phenotype (24, 25). However, as the D1R/D5R antagonist used in these studies is also an agonist for serotonin receptors (26, 27) and serotonin receptors are also expressed on DCs (28), the individual contributions of specific DARs and serotonin receptors in this phenomenon are not clear (24).

The involvement of Th1/Th17 T cells and Tregs in autoimmunity and the possible connection between type I DAR signaling in DCs and the biasing of T cell fate suggest that type I DARs expressed on immune cells are involved in the balance between autoimmunity and tolerance. Notably, alterations in components of the dopaminergic system have been correlated with multiple sclerosis (MS), an inflammatory and demyelinating neurodegenerative disease of the CNS mediated mainly by Th17 and Th1 autoreactive T cells. In this regard, patients suffering from MS show decreased expression of D5R in PBMCs compared with that in healthy individuals (29). Furthermore, increased local levels of DA in the CNS were detected in mice induced with experimental autoimmune encephalomyelitis (EAE), an animal model of MS, compared with those in controls (30). Taken together, this evidence suggests a possible modulatory role for DA via type I DARs in this autoimmune disease. However, further efforts are necessary to identify specific receptors and cells involved in DA-mediated regulation of this autoimmune response.

In this study, we investigate the contribution of DA in modulating the pivotal role of DCs during adaptive immune response. We report that DCs express several DARs and the components required to synthesize, to store, and to degrade DA. Our data suggest an autocrine modulatory role of DA in DC function and illustrate that DA stimulates D5R expressed on DCs thereby facilitating strong $CD4^+$ T cell activation and differentiation toward the Th17 phenotype, thus contributing to the development of a $CD4^+$ T cell-mediated autoimmune response.

Materials and Methods

Animals

Six- to eight-week-old mice of the C57BL/6 background were used for all experiments. Wild-type (WT) C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). D5R-knockout (D5RKO) mice were kindly donated by Dr. David Sibley (31). OVA-specific OT-I transgenic mice expressing specific TCRs for $H\text{-}2K^b/OVA_{257–264}$ were obtained from Taconic. OVA-specific OT-II transgenic mice expressing specific TCRs for $I\text{-}A^b/OVA_{323–339}$ were kindly donated by Dr. María Rosa Bono (32). All mice were maintained and manipulated according to institutional guidelines at the pathogen-free facility of the Fundacion Ciencia y Vida.

Generation of DCs

Bone marrow-derived DCs from WT and D5RKO mice were prepared as previously described (33). Briefly, DCs were grown in RPMI 1640 medium (Hyclone, Logan, UT) supplemented with 5% heat-inactivated FBS (Biological Industries, Beit Haemek, Israel) and 10 ng/ml recombinant mouse GM-CSF (PeproTech, Rocky Hill, NJ). On day 5, differentiation of DCs was routinely assessed obtaining >80% $CD11c^+$ cells. In some experiments, day 5 DCs were either left unstimulated (immature dendritic cells; iDCs) or stimulated (mature dendritic cells; mDCs) with 100 ng/ml LPS (Sigma Chemical Co., St. Louis, MO) for 24 h and used for further experiments.

Immunostaining and Western blots

To determine expression levels of key surface molecules, DCs were immunostained with the following fluorochrome-conjugated mAbs for 30 min: allophycocyanin-conjugated anti-CD11c (clone HL3), PE-conjugated $anti\text{–}I\text{-}A^b$ (clone AF6-120.1), FITC-conjugated anti-CD80 (clone 16-10A1), PE-conjugated anti-CD86 (clone GL1), FITC-conjugated $anti\text{–}H2\text{-}K^b$ (clone AF6-88.5), and FITC-conjugated anti-CD40 (clone 3/23), all of them from BD Pharmingen (San Diego, CA). For DAR detection, DCs were incubated with unconjugated, rabbit anti-D2R, rabbit anti-D3R, or goat anti-D4R polyclonal Abs (pAbs) specific for extracellular epitopes for 1 h, followed by FITC-conjugated anti-rabbit IgG or anti-goat IgG Abs (Santa Cruz Biotechnology, Santa Cruz, CA). In the case of D1R-like receptor and TH detection, Abs against intracellular epitopes were used. Accordingly, DCs were fixed with 1% paraformaldehyde in PBS for 15 min at room temperature and then treated with permeabilizing buffer (0.5% saponin, 3% BSA in PBS). Permeabilized cells were incubated with unconjugated rabbit anti-D1R, rabbit anti-D5R, or rabbit anti-TH pAbs, followed by FITC-conjugated anti-rabbit IgG Abs (Santa Cruz Biotechnology). Nonspecific Ig was included as control in each case, and staining was analyzed by flow cytometry in the $CD11c^+$ $I\text{-}A^{b+}$ population. For intracellular cytokine staining, cells were stimulated for 4 h with PMA (50 ng/ml; Sigma) and ionomycin (1 μg/ml; Sigma) in the presence of brefeldin A (5 μg/ml; Sigma). After staining of surface markers, cells were fixed, permeabilized, and incubated for 30 min with allophycocyanin-conjugated anti–IFN-γ (clone XMG1.2) and PE-conjugated anti–IL-17 mAbs (clone TC11-18H10), both from BD Pharmingen. All flow cytometry analyses were performed by using a FACSCanto II flow cytometer, and collected data were analyzed by using FACSDiva software (both from BD Biosciences). To determine DAT expression, DCs were purified using CD11c microbeads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), and DAT expression was analyzed in whole-cell extracts by immunoblot using a rat anti-DAT mAb (clone 6-5G10) and an HRP-conjugated secondary anti-rat IgG Ab (both from Santa Cruz Biotechnology). Immunodetection of β-actin was included as a loading control, using mouse anti–β-actin mAb (clone AC-15; Sigma) and an HRP-conjugated anti-mouse IgG Ab (Rockland, Gilbertsville, PA).

RT-PCR

Cells and tissues were lysed, and total RNA was extracted with the EZNA total RNA kit (Ω Bio-Tek, Norcross, GA), treated with DNase using TURBO DNA-free kit (Ambion, Austin, TX), and 1 μg RNA was retrotranscribed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer instructions. RT-PCR was performed using 1 μl cDNA (equivalent to 100 ng), 10 μl Go-Taq Green Master Mix 2× reagent (Promega, Madison, WI), and primers and water for a final volume of 20 μl. Forward and reverse primers were used at 0.5 μM. PCR was carried out for 35 cycles with 95°C melting (30 s), 57°C annealing (45 s), and 72°Cextension (45 s). Primer sequence were as follows: β-actin forward, 5′-CAGCTTCTTTGCAGCTCCTT-3′; β-actin reverse, 5′-CCTGGATGGCTACGTACATGGC-3′; serotonin transporter (SERT) forward, 5′-CTGAGATGAGGAACGAAGAC-3′; SERT reverse, 5′-CTGAGTGATTCCATAGAACCA-3′; norepinephrine transporter (NET) forward, 5′-GCTAGATAGTTCAATGGGAGG-3′; NET reverse, 5′-CTCACGAACTTCCAACACAG-3′; MAO-A forward, 5′-GCATGATAATTGAAGATGAGGAGG-3′; MAO-A reverse, 5′-CGAATCACCCTTCCATACAG-3′; MAO-B forward, 5′-GTATGGAATCCTATCACCTACC-3′; MAO-B reverse, 5′-AATTTCCTCTCCTGTCCTCC-3′; DβH forward, 5′-TTCCAATGTGCAGCTGAGTC-3′; DβH reverse, 5′-GGTGCACTTGTCTGTGCAGT-3′; VMAT2 forward, 5′-TGCCAGCGAGCATCTCTTAT-3′; VMAT2 reverse, 5′-CTTCCTTAGCAGGTGGACTT-3′. Amplicon sizes were verified by electrophoresis on a 1.5% agarose gel after ethidium bromide staining.

Analysis of cytokine mRNA production by real-time RT-PCR

Cells were lysed and RNA was extracted using the TRIzol reagent. cDNA (1 μg) was synthesized using oligonucleotide and M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. A 20-μl real-time PCR reaction included 1 μl cDNA, 10 μl Brilliant SYBR Green

QRT-PCR Master Mix (Stratagene), and primers and water as indicated by the manufacturer's instructions. PCR was carried out for 40 cycles with 95°C melting (30 s), 60°C annealing (45 s), and 72°C extension (40 s). All reactions were performed on a Stratagene Mx3000P. Primer sequences were as follows: IL-23 forward, 5′-TGCTGGATTGCAGAGCAGTAA-3′; IL-23 reverse, 5′-GCATGCAGAGATTCCGAGAGA-3′; IL-6 forward, 5′-AGGATACCACTCCCAACAGACCT-3′; IL-6 reverse, 5′-CAAGTGCATCATCGTTGTTCATAC-3′; IL-1β forward, 5′-CAAATCTCGCAGCAGCACA-3′; IL-1β reverse, 5′-TCATGTCCTCATCCTGGAAGG-3′; TGF-β forward, 5′-TGCGCTTGCAGAGATTAAAA-3′; TGF-β reverse, 5′-CTGCCGTACAACTCCAGTGA-3′; GADPH forward, 5′-TCCGTGTTCCTACCCCCAATG-3′; GADPH reverse, 5′-GAGTGGGAGTTGCTGTTGAAG-3′. For relative quantification, mRNA expression in each sample was normalized by comparison with the GAPDH mRNA expression using the ddCT method as previously described (34).

Phosphorylation of ERKs

DCs were incubated with FBS-free medium for 14 h before experiments. DCs (2×10^6 cells/ml) were washed twice and resuspended in prewarmed medium either in the presence or absence of 100 ng/ml LPS and either left untreated or treated with 1 nM SKF38393 (SKF) or 1 nM SCH23390 (SCH; Tocris, Bristol, U.K.) for 10 min. Cells were lysed with ice-cold lysis buffer 2× [2% Triton X-100, 100 mM Tris-HCl pH 7.6, 80 mM β-glycerophosphate, 50 mM sodium fluoride, 2 mM sodium orthovanadate and protease inhibitor mixture (Sigma)]. Cell lysates (50 μg/sample) were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Hybond-P; Amersham Biosciences, Uppsala, Sweden), and diphosphorylated-ERK1/2 was detected using a mouse phospho-specific ERK1/2 mAb (1:2000; Sigma) followed by HRP-conjugated goat anti-mouse IgG Ab (1:2000; Rockland). Immunodetection was carried out with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). Membranes were stripped and reprobed with rabbit anti-ERK1/2-specific pAb (1:30,000; Sigma) followed by HRP-conjugated goat anti-rabbit Ab (1:4000; Rockland) and detected as described above.

Cytokine ELISA

Levels of IL-6, IL-10, and IL-12 released into the culture supernatant of iDCs and mDCs and IL-2 secreted by T cells into the coculture supernatant were quantified by ELISA as previously described (35). Recombinant murine cytokines were used as standards for quantification. All of the reagents, recombinant cytokines, and Abs used were purchased from BD Pharmingen. When indicated, DCs were pretreated with 1 μM reserpine (Tocris) for 1 h to promote depletion of intracellular stores of DA. Subsequently, cells were washed, resuspended in fresh prewarmed medium, and stimulated or not with LPS as described earlier.

T cell activation assays

iDCs or mDCs were washed and resuspended in fresh prewarmed medium. Subsequently, DCs were cocultured at indicated ratios (see the figures and figure legends that accompany this article) with either purified OT-I or OT-II T cells (10^5 T cells/well) in the presence of 0.1 ng/ml $OVA_{257–264}$ peptide or 200 ng/ml $OVA_{323–339}$ peptide (GenScript, Piscataway, NJ), respectively. Purification of OT-I and OT-II T cells from total splenocytes was carried out by negative selection using $CD8^+$ or $CD4^+$ T cell isolation kits (Miltenyi Biotec), respectively. T cell activation was determined as IL-2 secretion in the coculture supernatant after incubation for 24 h by ELISA (35). For determination of T cell proliferation, purified OT-I or OT-II T cells were stained with 5 μM CFSE (Invitrogen) in the presence of 5% heat-inactivated FBS for 5 min at room temperature. Subsequently, cells were washed and cocultured with DCs as indicated earlier. After 72 h of coculture incubation, cells were stained either with allophycocyanin-conjugated anti-CD8 (clone 53-6.7) or anti-CD4 (clone RM4-5) (both from BD Pharmingen), and CFSE-associated fluorescence of the $CD8^+$ or $CD4^+$ populations were analyzed by flow cytometry.

EAE induction and evaluation

Six- to eight-week-old female C57BL/6 WT or D5RKO mice were injected s.c. with 50 μg myelin oligodendrocyte glycoprotein 35–55 peptide (pMOG; Genetel Laboratories, Madison, WI) emulsified in CFA (Invitrogen) supplemented with heat-inactivated *Mycobacterium tuberculosis* H37 RA (Difco Laboratories, Detroit, MI). In addition, mice received i.p. injections of 500 ng pertussis toxin (Calbiochem, La Jolla, CA) on days 0 and 2. Clinical signs were assessed daily according to the following scoring criteria: 0, no detectable signs; 1, flaccid tail; 2, hind limb weakness or abnormal gait; 3, complete hind limb paralysis; 4, paralysis of fore and hind limbs; and 5, moribund or death. In some EAE experiments, 10^6 bone marrow-derived DCs from WT and D5RKO mice were pulsed with 5 μg/ml pMOG for 4 h and then transferred i.v. into WT C57BL/6 recipient mice 14 and 7 d before EAE induction. For the preparation of CNS mononuclear cells, mice were perfused through the left cardiac ventricle with cold PBS. The brain and spinal cord were dissected, and CNS tissue was cut into small pieces and digested by collagenase D (2.5 mg/ml; Roche Diagnostics) and DNaseI (1 mg/ml; Sigma) at 37°C for 45 min. Digested tissue was passed through a 70-μm cell strainer obtaining single-cell suspension that was subjected to centrifugation in a Percoll gradient (70%/37%). Mononuclear cells were removed from the interphase and resuspended in culture medium for further analysis.

Statistical analysis

Statistical significance of differences between groups was evaluated by two-tailed Student *t* test or Mann–Whitney rank sums two-tailed *U* test by using GraphPad Prism software.

Results

DCs express functional D5R whose expression is modulated during maturation

To determine whether DA could regulate DC function, we first evaluated the expression of DARs on the cell surface using specific Abs and flow cytometry. Our results show that both iDCs and mDCs express D1R, D2R, D3R, and D5R. Notably, only D5R expression was significantly downregulated on DCs after LPS treatment (Fig. 1A, 1B), suggesting that D5R could be involved in the regulation of DC function during maturation. Inflammatory signals triggered by TLR stimulation on DCs involve activation of several signaling pathways including NF-κB, MAPKs, PI3K, and STATs (36). To test whether D5R engagement is capable of affecting LPS-induced intracellular signaling, we studied the phosphorylation of key signaling molecules involved in cytokine production, ERK1/2, JNK, and p38 MAPK (37). Stimulation with the selective D1R/D5R agonist SKF attenuated LPS-induced ERK1/2 phosphorylation in WT DCs (Fig. 1C, *top panel*), an effect not observed in D5RKO DCs (Fig. 1C, *bottom panel*). No significant differences were observed in levels of ERK1/2 phosphorylation when WT or D5RKO DCs were treated with the D1R/D5R antagonist SCH in the presence (Fig. 1C) or absence of LPS (data not shown). No changes in phosphorylation levels of p38 and JNK were detected upon stimulation of D1/D5 receptors in WT or D5RKO DCs (data not shown). These results indicate that D5R is expressed on DCs, and its stimulation is coupled to modulation of LPS-triggered signaling pathways.

DCs express the machinery to synthesize, store, and degrade DA

Previous studies have shown that some immune cells not only express neurotransmitter receptors but also have the ability to synthesize, internalize, and accumulate neurotransmitters in intracellular reservoirs (38, 39). Such properties enable these cells to release neurotransmitters in the presence of certain stimuli. These neurotransmitters may act in an autocrine/paracrine manner to modulate DC function (37, 40). We investigated the expression of key enzymes necessary for biosynthesis, degradation, uptake, and storage of DA in DCs. Accordingly, we first analyzed intracellular TH expression in DCs, an enzyme necessary to synthesize DA. We found that DCs express TH and that its expression is regulated during DC maturation (Fig. 2A). Next, transcripts for VMAT2, which mobilizes cytosolic DA toward vesicular storage, were detected in iDCs, whereas very low to undetectable levels were found in mDCs (Fig. 2B). These results correlate with the decreased expression of TH detected in mDCs compared with that in iDCs (Fig. 2A). In contrast, expression of DβH mRNA, an enzyme that oxidizes DA to produce norepinephrine in catecholaminergic cells, could not be detected in either iDCs or mDCs

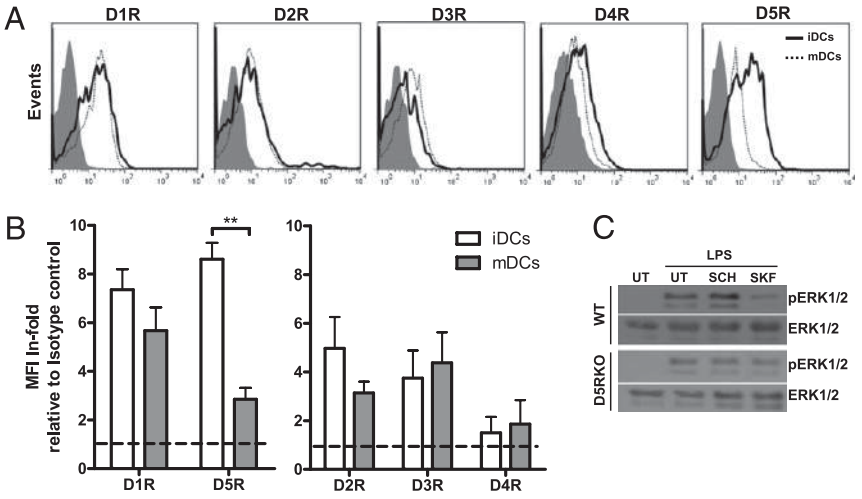

FIGURE 1. Expression of functional D5R in DCs. DCs obtained from WT mice were either left unstimulated (iDCs) or stimulated with 100 ng/ml LPS for 48 h (mDCs), and DAR expression was assessed in the $CD11c^+$ $I\text{-}A^{b+}$ population by flow cytometry (**A**, **B**). (A) Representative histograms of DAR expression in iDCs (bold line) and mDCs (dotted line) are shown. Filled histograms correspond to nonspecific Ig control for each receptor subtype. (B) The ratio of the mean fluorescence intensity (MFI) corresponding to specific label over MFI corresponding to unspecific control is represented. Data from four independent experiments are shown. Values represent mean ± SD. $^{**}p < 0.01$ (unpaired Student *t* test). (**C**) DCs obtained from WT or D5RKO mice were either left untreated (UT) or treated with 100 ng/ml LPS, alone or in the presence of either the D1R/D5R agonist SKF or the D1R/D5R antagonist SCH for 10 min. Cells were lysed in the presence of phosphatase inhibitors, and the presence of diphosphorylated ERK1/2 (pERK1/2, *top panel*) or total ERK1/2 irrespective of their phosphorylations (ERK1/2, *bottom panel*) was analyzed in protein extracts by Western blot. Representative data from one of three independent experiments are shown.

(Fig. 2B). Thus, DCs express some components required to synthesize and store DA but do not seem to contain components to convert DA to norepinephrine.

Some dopaminergic cells can also take up DA from the extracellular environment. According to this notion, we analyzed the expression of DAT and NET, which can both take up DA from the extracellular compartment. Neither DAT protein (Fig. 2C) nor NET mRNA (Fig. 2D) were detected in DCs. The human and rat SERT were recently described to mediate DA uptake (41). Notably, SERT mRNA expression was found in both iDCs and mDCs (Fig. 2D), suggesting that DCs could take up DA in a noncanonical fashion. Next, we assessed the expression of DA-degrading enzymes in DCs. We detected the mRNA expression of two monoamine oxidase enzymes, MAO-A and MAO-B, in both iDCs and mDCs (Fig. 2E), indicating that DCs are capable of catabolizing intracellular DA. Taken together, these results indicate that DCs express molecular components required to synthesize, store, and degrade DA.

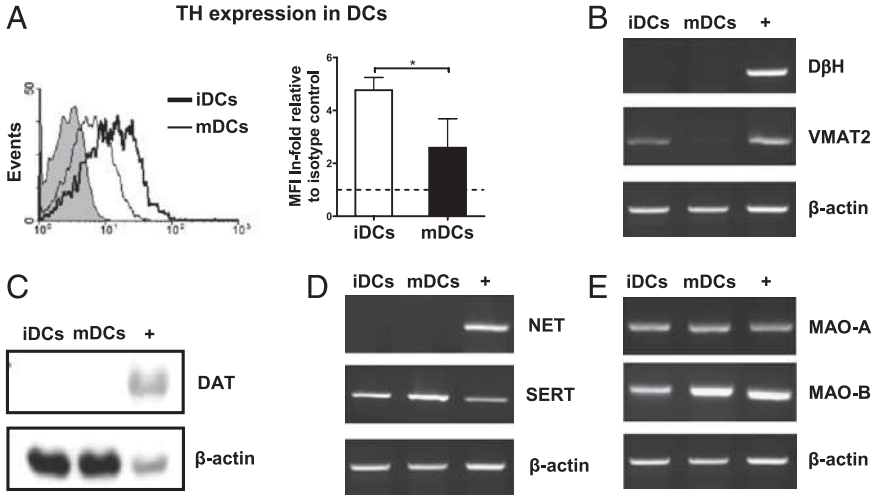

FIGURE 2. Expression of components of the dopaminergic system in DCs. Bone marrow-derived DCs were either left unstimulated (iDCs) or stimulated with LPS (mDCs). After 24 h, cells were analyzed for protein expression by flow cytometry (**A**) or Western blot (**C**) and for mRNA expression by RT-PCR (**B**, **D**, and **E**). (A) Intracellular TH expression was analyzed in the $CD11c^+$ $I\text{-}A^{b+}$ population. In the *left panel*, a representative result is shown. Isotype control is indicated with a solid gray area. In the *right panel*, the ratio of MFI corresponding to specific label over MFI corresponding to isotype control is represented. Data from four independent experiments are shown. Values represent mean ± SD. $^{*}p < 0.05$ (unpaired Student *t* test). (B) Expression of enzymes involved in catecholamine biosynthesis (DβH) and storage (VMAT2) was assessed with specific primers. (C) Detection of the plasma membrane DAT was assessed in total cell extracts by using specific Abs. (D) Transcripts of norepinephrine and serotonin transporters were also analyzed using NET and SERT specific primers, respectively. (E) Transcripts encoding for DA degrading enzymes, MAO-A and MAO-B, were detected by using specific primers. Representative data from two experiments are shown. Positive controls (+) correspond to suprarenal gland, except for SERT, for which cerebellum was used. β-Actin was used as loading control.

D5R selectively modulates cytokine secretion by LPS-stimulated DCs

Next, we determined whether D5R regulates the phenotype of DCs upon maturation. To do this, we evaluated the secretion of regulatory cytokines and expression of key surface markers by WT and D5RKO DCs either untreated or matured with LPS. Whereas no differences were detected in the levels of IL-12, IL-10, and IL-6 secreted by WT or D5RKO iDCs, D5RKO mDCs secreted significantly less IL-12 compared with that of WT mDCs (Fig. 3A). We observed no differences in levels of IL-10 and IL-6 released by WT or D5RKO mDCs. To gain more insight into the role of D5R in the production of other important cytokines involved in $CD4^+$ T cell polarization, we determined mRNA levels for IL-23, TGF-β, and IL-1β produced by WT or D5R-deficient DCs. Results show that expression of D5R contributed significantly to the production of IL-23 mRNA by mDCs but not by iDCs (Fig. 3B). Conversely, D5R was not relevant for production of TGF-β or IL-1β by DCs (Fig. 3B). A comparison of the expression of surface maturation markers such as $I\text{-}A^b$, CD80, and CD86 in WT and D5RKO mDCs revealed no differences (Supplemental Fig. 1). Given that DCs express machinery to synthesize and store DA, we hypothesized that intracellular stores of DA may stimulate D5R in an autocrine manner and promote IL-12 stimulation upon maturation. To test this possibility, we performed LPS-induced maturation experiments in which WT DCs were previously depleted of intracellular stores of DA by treatment with reserpine (42). Depletion of DA caused a significant decrease in IL-12 secretion in mDCs without affecting IL-10 or IL-6 production (Supplemental Fig. 2). These results suggest that during the maturation process, DC-derived DA selectively facilitates secretion of some regulatory cytokines by stimulating the D5R in an autocrine manner.

D5R expressed on DCs facilitates a strong $CD4^+$ T cell response

Cytokines play an important role in the priming of T cells by DCs. Because we found that D5R selectively regulates cytokine production in DCs (Fig. 3), we assessed the ability of D5R expressed on DCs to modulate the priming of naive Ag-specific T cells. To this end, OVA-specific naive T cells were cocultured with WT or D5RKO DCs pulsed with OVA-derived peptides (see *Materials and Methods*), and T cell activation and proliferation were determined by measuring IL-2 secretion and dilution of CFSE-associated fluorescence, respectively. Whereas no difference in IL-2 release was observed from $CD4^+$ T cells cocultured with WT or D5RKO iDCs, $CD4^+$ T cells produced much less IL-2 when cultured with D5R-deficient mDCs than when cultured with WT mDCs (Fig. 4A). This suggests that D5RKO mDCs are significantly less efficient at activating $CD4^+$ T cells than are WT mDCs. In contrast, no difference was observed in IL-2 production from $CD8^+$ T cells when cocultured with WT or D5RKO DCs, regardless of the maturation state (Fig. 4A).

Because differences in the capability of DCs to promote efficient T cell activation (Fig. 4A) and cytokine production (Fig. 3) were observed between WT and D5R-deficient DCs only at the mature state, we next compared T cell proliferation induced by WT or D5RKO mDCs. In support of our previous results, we found that D5R-deficient mDCs were less efficient at inducing $CD4^+$ T cell proliferation compared with WT mDCs (Fig. 4B). We observed no differences in the proliferation rates of $CD8^+$ T cells when they were cocultured either with WT or D5RKO mDCs (Fig. 4B), confirming our data of $CD8^+$ T cell activation (Fig. 4A). These results indicate that D5R expressed in DCs facilitates strong priming of $CD4^+$ T cells but does not contribute to $CD8^+$ T cell activation or proliferation.

Absence of D5R on DCs decreases EAE severity

To study the relevance of the D5R in the modulation of the $CD4^+$ T cell-mediated response in vivo, we used EAE, a murine model of autoimmunity mainly mediated by autoreactive $CD4^+$ T cells (43). Accordingly, we first compared the susceptibility of WT and D5RKO mice to develop the disease after EAE induction. Disease onset and progression were quantified using a clinical score as described in *Materials and Methods*. Whereas there were no differences in disease incidence between WT and D5RKO mice, D5R-deficient mice displayed a delayed onset of the disease compared with WT mice (day 12.80 ± 2.37 versus day 9.54 ± 2.18, $p < 0.001$) (Fig. 5A). Additionally, D5RKO mice exhibited a significantly reduced clinical score or severity of disease compared with that of WT mice (Fig. 5A).

To determine the contribution of the D5R expressed specifically on DCs to EAE development and progression, pMOG-pulsed WT or D5R-deficient DCs were transferred into WT recipient mice

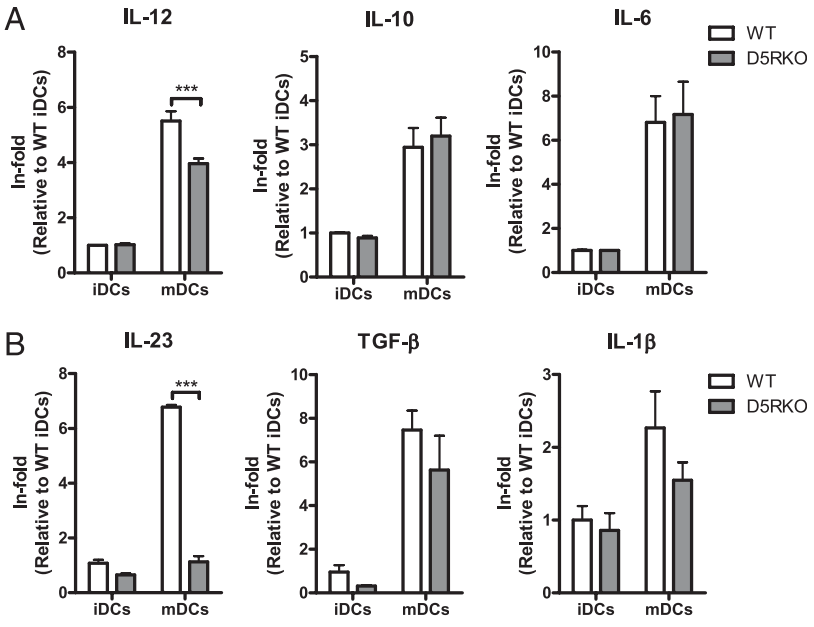

FIGURE 3. Lack of D5R selectively impairs cytokine production on LPS-stimulated DCs. (**A**) DCs obtained from WT (white) or D5RKO (gray) mice were either left unstimulated (iDCs) or stimulated with 100 ng/ml LPS (mDCs). After 24 h, IL-12, IL-10, and IL-6 secretion was evaluated by ELISA (A), and IL-23, TGF-β, and IL-1β mRNA expression was evaluated by semiquantitative real-time RT-PCR (**B**). Data correspond to the fold increase in cytokine secretion (A) or cytokine mRNA expression (B) relative to WT iDCs. PCR amplification of GADPH was used as an internal control (B). Values represent mean ± SD from at least three independent experiments. $***p < 0.001$ (unpaired Student *t* test).

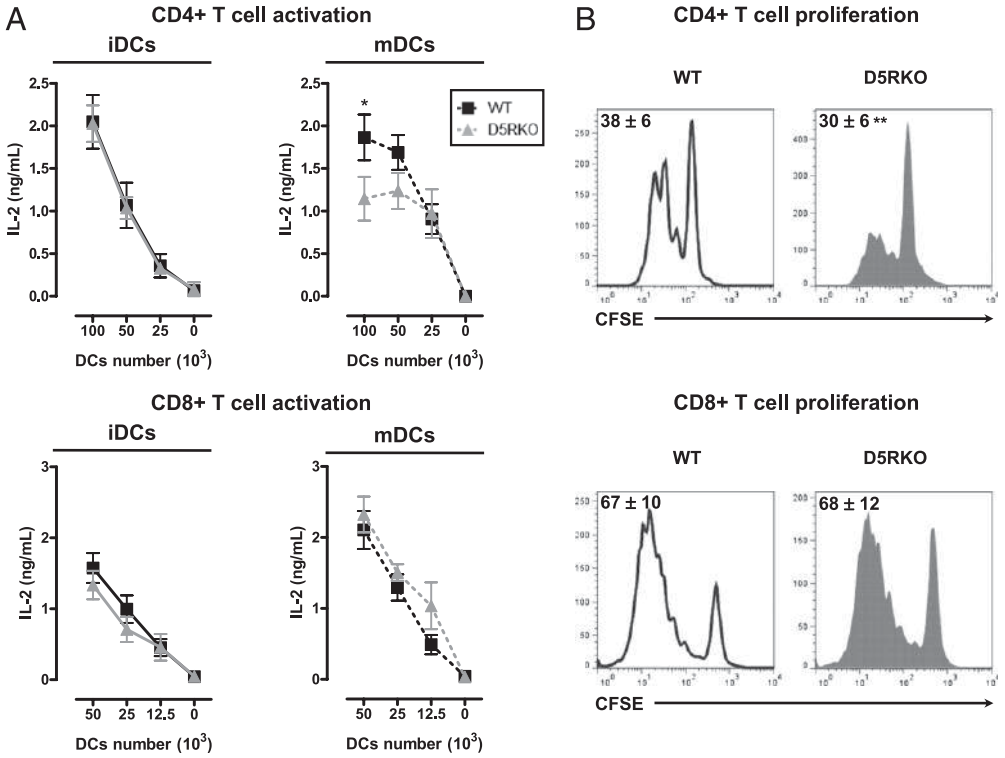

FIGURE 4. DCs lacking D5R show impaired $CD4^+$, but not $CD8^+$, T cell priming ability. (**A**) DCs obtained from WT or D5RKO mice were either left unstimulated (iDCs) or stimulated with LPS (mDCs) for 24 h and subsequently cocultured with $CD4^+$ (*top panels*) or $CD8^+$ (*bottom panels*) T cells purified from OT-II or OT-I mice, respectively, in the presence of corresponding OVA-derived peptides. After 24 h, IL-2 release was assessed in the culture supernatant by ELISA. Data from four independent experiments are shown. Values represent mean ± SEM. $*p < 0.05$ (unpaired Student *t* test). (**B**) DCs obtained from WT or D5RKO mice were stimulated with LPS (mDCs) for 24 h and then cocultured with CFSE-stained $CD4^+$ (*top panels*) or $CD8^+$ (*bottom panels*) T cells purified from OT-II or OT-I mice (5×10^3 DCs and 10^5 T cells/well), respectively, in the presence of corresponding OVA-derived peptides. After 72 h, dilution of CFSE-associated fluorescence was assessed in the $CD4^+$ or $CD8^+$ population by FACS. Numbers on histograms represent percentage of proliferating cells. Data are representative of at least four independent experiments. Values represent mean ± SEM. $**p < 0.01$ (compared with WT by paired Student *t* test).

14 and 7 d before EAE induction, and disease progression was monitored daily. We found that mice that received D5RKO DCs presented a significantly less severe disease than mice that received WT DCs (Fig. 5B). There were no significant differences in the disease incidence or kinetics of disease onset when comparing the development of EAE on WT DC- or D5RKO DC-recipient mice (data not shown).

Because of the pivotal role of CNS-infiltrating Th1, Th17, and Treg $CD4^+$ T cells in the outcome of EAE (43), we examined the functional phenotypes of $CD4^+$ T cells infiltrating the CNS at the peak of the disease. At day 15 after disease induction, we isolated the infiltrating mononuclear fraction from spinal cords and brains of mice transferred with either WT or D5R-deficient DCs (see *Materials and Methods* for more details). No differences were detected in the levels of infiltrating Tregs ($CD25^+$ $Foxp3^+$) or effector $CD4^+$ T cells ($CD25^+$ $Foxp3^-$) (data not shown). However, we did detect significant differences in the levels of $CD4^+$ T cell subtypes (for gating strategy, see Supplemental Fig. 3). We found a significant reduction in the levels of IL-17–producing $CD4^+$ T cells in the CNS of mice that received D5RKO DCs compared with those in the CNS of mice that received WT DCs (Fig. 5C, 5D). The proportion of IFN-γ^+ $CD4^+$ T cells were variable between the two groups, but no significant differences were found (Fig. 5C, 5D). Notably, the percentage of double-positive IFN-γ^+ IL-17^+ $CD4^+$ T cells infiltrating the CNS was significantly reduced in mice that received D5RKO DCs compared with those in the CNS of mice that received WT DCs (Fig. 5C, 5D). Because DA has also been involved in the polarization of $CD4^+$ T cells toward Th2 phenotype (25), we evaluated the presence of IL-4^+ cells in the population of $CD4^+$ T cells infiltrating the CNS. We did not detect IL-4–producing cells in the CNS of mice receiving WT or D5RKO DCs (data not shown), thereby discarding the participation of Th2 cells. Taken together, these data indicate that D5R expressed on DCs is able to modulate the development of an autoimmune response in vivo by biasing the differentiation of $CD4^+$ T cells toward the Th17 phenotype.

Discussion

In this work, we present data from in vitro experiments as well as an in vivo autoimmunity model that illustrate the involvement of D5R signaling in DCs in the determination of $CD4^+$ T cell fate. Our data from in vitro experiments suggest a mechanism triggered by DC maturation, which involves DA secretion and subsequent autocrine stimulation of D5R promoting selective regulation on cytokine release, thereby contributing to efficient Ag-specific $CD4^+$ T cell response. In agreement with our in vitro data, in this study we show that D5R expressed on DCs contributes to the development of a $CD4^+$ T cell-driven autoimmunity. These findings contribute to the knowledge of DC physiology and suggest relevant molecular targets for immunotherapy.

Accumulating evidence has pointed to a role for neurotransmitters in regulating the immune system and influencing the activation and differentiation of T cells. In agreement with this, we show that mouse DCs express receptors for the neurotransmitter DA and express components necessary to synthesize, store, and degrade DA itself. The timing of expression of these components

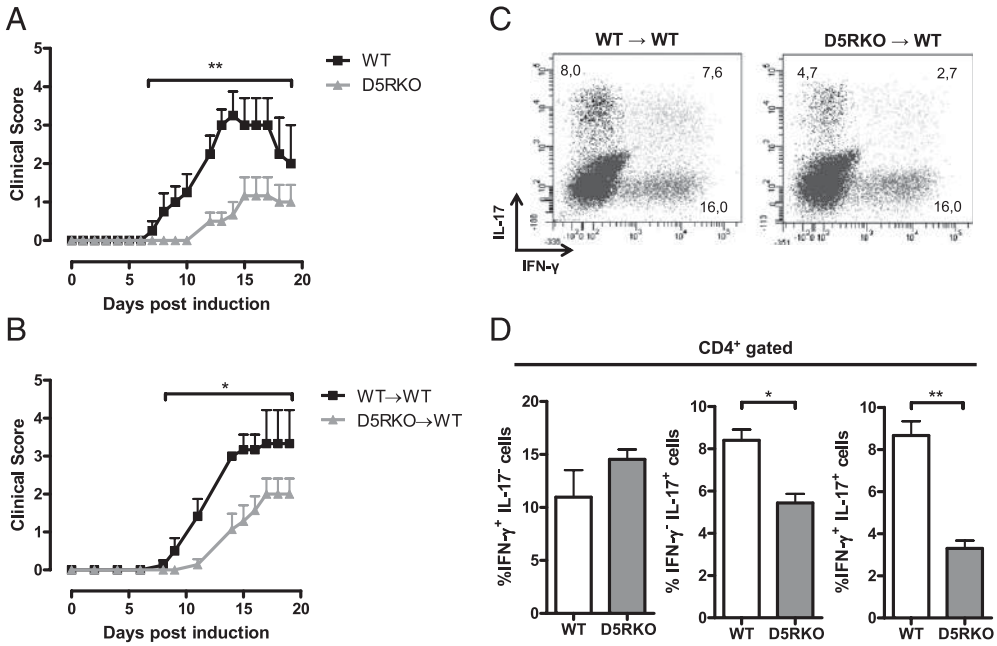

FIGURE 5. The absence of D5R on DCs reduces severity of EAE by decreasing infiltration of IL-17–producing $CD4^+$ T cells into the CNS. (**A**) EAE was induced in WT mice (black line) and D5RKO mice (gray line) by immunization with pMOG in CFA followed by pertussis toxin injection (see *Materials and Methods*). (**B**) WT (black line) or D5RKO (gray line) purified bone marrow-derived DCs were pulsed with pMOG and transferred (10^6 DCs/mice; i.v. injections) into WT recipient mice at days 14 and 7 prior to EAE induction. Disease severity was evaluated as clinical score (see *Materials and Methods*) from day 0 to day 20 postinduction (A, B). Data from six to eight mice in each group, corresponding to a representative from three (A) and two (B) independent experiments, are shown. Values represent mean ± SEM. $*p < 0.05$, $**p < 0.01$ (Mann–Whitney *U* test). Mononuclear cells were isolated from CNS followed by ex vivo stimulation with PMA/ionomycin in the presence of brefeldin A, and intracellular cytokine staining analysis was carried out by flow cytometry. (**C**) Representative dot plots for IL-17 versus IFN-γ production in the infiltrating $CD4^+$ gated population are shown. Numbers at the corners indicate the percentage of IFN-γ^+ IL-17^-, IFN-γ^+ IL-17^+, and IFN-γ^- IL-17^+ cells, respectively. (**D**) Bar graphs indicate percentage of CNS-infiltrating $CD4^+$ T cells producing IFN-γ (*left panel*), IL-17 (*central panel*), or both cytokines (*right panel*). Data representative of two independent experiments are shown. Values represent mean ± SEM. $*p < 0.05$, $**p < 0.01$ (unpaired Student *t* test).

(Fig. 2) and the requirement for intracellular DA to induce selective cytokine secretion from mDCs (Supplemental Fig. 2) suggest an autocrine mechanism of action of this neurotransmitter. These results are in agreement with previous observations that an anti-DA Ab produced positive immunoreactivity in human monocyte-derived DCs (25). Notably, only D5R expression decreased upon LPS-induced DC maturation, suggesting a role for this receptor during DC maturation. Using a genetic approach, we were able to analyze the particular effects of D5R on DC function. In cell culture, D5R appears to be important for both IL-23 and IL-12 production from mature mDCs and the subsequent activation of $CD4^+$ T cells.

A previous study hinted at the involvement of type I DARs in the amelioration of EAE, the murine model of MS. In this study, the systemic treatment of mice with the type I DAR antagonist SCH alleviates the development of EAE by decreasing the Th17 response (24). However, this pharmacologic approach does not permit discrimination between the effects of D5R or D1R because the drug inhibits both type I DARs. In fact, SCH not only displays similar affinities for D1R and D5R (K_i = 0.2 and 0.3 nM, respectively) (44) but also displays comparable affinities for serotonin receptors 5-HT1C and 5-HT2C (K_i = 6.3 and 9.3 nM, respectively) (26, 27). Furthermore, this study could not confine the cell type responsible for the amelioration effect. EAE is mediated mainly by T cells, but B cells also contribute to the initiation and development of the disease (43, 45, 46). Type I DARs as well as serotonin receptors have been found to be expressed on many types of immune cells including DCs (47), T cells (23, 48, 49), and B cells (50, 51). Therefore, systemic treatment with SCH could affect several types of immune cells. To restrict the contribution of DC signaling to the amelioration of EAE, the authors treated DCs with SCH ex vivo and transferred treated cells into WT recipient mice (24). A slight decrease in EAE severity was seen in mice transferred with SCH-treated DCs, but such differences were not statistically significant (24).

In contrast, our genetic approach allowed us to determine the contribution of DCs and specific DARs to $CD4^+$ T cell differentiation and EAE development and progression. By comparing results obtained from WT DCs and D5RKO DCs, we were able to determine the effects of D5R signaling on DCs and to resolve the contributions of D5R signaling to $CD4^+$ T cell polarization. We found that D5R signaling in DCs selectively affects IL-23 and IL-12 production by DCs (Fig. 3) and contributes to $CD4^+$ T cell activation and proliferation (Fig. 4). In our in vivo model of EAE, we showed that signaling through D5R on DCs is important for the development of this disease. D5R-deficient mice exhibited delayed EAE progression with reduced severity compared with normal mice (Fig. 5), presumably due to the reduced proportion of Th17 (IL-17^+) T cells present in the CNS of these mice (Fig. 5). Thus, although data are in agreement with a previous study (24), we were able to show that D5R specifically expressed on DCs contributes directly to $CD4^+$ T cell fate and disease progression. In addition to the decreased disease severity, we also observed that the onset of EAE was delayed in D5RKO mice compared with that in WT mice (Fig. 5A). This delay was not seen in WT mice transferred with D5RKO DCs (Fig. 5B). Moreover, the decrease in EAE severity was not as robust in WT mice transferred with D5RKO DCs (Fig. 5B) as that observed in D5RKO mice (Fig. 5A). These differences in the decrease of EAE severity and in disease onset observed in D5RKO mice and WT mice transferred

with D5RKO DCs may suggest that D5R signaling in cell types other than DCs is involved in the progression and development of this autoimmune disease. In this regard, Kipnis et al. (52) have described that Tregs express type I DARs, and stimulation of these receptors attenuates the suppressive activity of Tregs. Thus, absence of D5R in Tregs could favor the suppressive activity of these cells and thereby contribute to decreased EAE manifestation observed in D5RKO mice (Fig. 5A), which should not be observed in WT mice transferred with D5RKO DCs (Fig. 5B).

Another nonexcluding possibility for explaining the differences on EAE severity and onset between D5RKO mice and WT mice transferred with D5RKO DCs is that endogenous DCs in WT recipients could compete with exogenously administered D5RKO DCs. Some of the exogenously administered D5RKO DCs bearing pMOG could die soon after injection and be phagocytosed by endogenous WT APCs. Thus, the effect due to the pMOG presentation by D5RKO DCs would be, in part, masked by pMOG presentation by WT APCs in vivo. Therefore, the occurrence of this possibility would lead to observation of a subestimated effect promoted by D5RKO DCs in WT mice (Fig. 5B) compared with that in D5RKO mice (Fig. 5A).

Considering the in vitro data, our data obtained from in vivo experiments show both expected and unexpected results. In agreement with the contribution of D5R expressed on DCs to the production of IL-23 observed in vitro (Fig. 3B), we observed that mice transferred with D5RKO DCs had fewer Th17 $CD4^+$ T cells infiltrating the CNS upon EAE (Fig. 5D). In contrast, despite IL-12 production from DCs being favored by D5R in vitro (Fig. 3), the proportion of CNS-infiltrating Th1 cells was not affected by D5R expressed on DCs in vivo (Fig. 5D). This discrepancy could be due to the presence of other mediators in vivo (but absent in vitro) that could act on DCs contributing to IL-12 production, making DA–D5R participation unnecessary and/or redundant for this effect. However, it seems that the DA–D5R axis operating in DCs is not redundantly contributing to strong IL-23 production in vivo.

In summary, the data presented in this study suggest the existence of an autocrine modulatory mechanism mediated by DA operating in DCs. DA release triggered by the DC maturation process could act through D5R and promote the release of proinflammatory cytokines. This could, in turn, induce a potent Th17 response in vivo, contributing to the development of autoimmunity.

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Disclosures

The authors have no financial conflicts of interest.

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