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Dopamine Receptor D3 Signaling on CD4$^+$ T Cells Favors Th1- and Th17-Mediated Immunity

Francisco Contreras,*,† Carolina Prado,* Hugo González,* Dafne Franz,* Francisco Osorio-Barrios,* Fabiola Osorio,‡,§ Valentina Ugalde,* Ernesto Lopez,¶ Daniela Elgueta,*,† Alicia Figueroa,* Alvaro Lladser,¶ and Rodrigo Pacheco*,†

Dopamine receptor D3 (DRD3) expressed on CD4$^+$ T cells is required to promote neuroinflammation in a murine model of Parkinson's disease. However, how DRD3 signaling affects T cell–mediated immunity remains unknown. In this study, we report that TCR stimulation on mouse CD4$^+$ T cells induces DRD3 expression, regardless of the lineage specification. Importantly, functional analyses performed in vivo using adoptive transfer of OVA-specific OT-II cells into wild-type recipients show that DRD3 deficiency in CD4$^+$ T cells results in attenuated differentiation of naive CD4$^+$ T cells toward the Th1 phenotype, exacerbated generation of Th2 cells, and unaltered Th17 differentiation. The reciprocal regulatory effect of DRD3 signaling in CD4$^+$ T cells favoring Th1 generation and impairing the acquisition of Th2 phenotype was also reproduced using in vitro approaches. Mechanistic analysis indicates that DRD3 signaling evokes suppressor of cytokine signaling 5 expression, a negative regulator of Th2 development, which indirectly favors acquisition of Th1 phenotype. Accordingly, DRD3 deficiency results in exacerbated eosinophil infiltration into the airways of mice undergoing house dust mite–induced allergic response. Interestingly, our results show that, upon chronic inflammatory colitis induced by transfer of naive CD4$^+$ T cells into lymphopenic recipients, DRD3 deficiency not only affects Th1 response, but also the frequency of Th17 cells, suggesting that DRD3 signaling also contributes to Th17 expansion under chronic inflammatory conditions. In conclusion, our findings indicate that DRD3-mediated signaling in CD4$^+$ T cells plays a crucial role in the balance of effector lineages, favoring the inflammatory potential of CD4$^+$ T cells. *The Journal of Immunology*, **2016, 196: 4143–4149.**

Microenvironmental cues present during CD4$^+$ T cell priming drive differentiation of various Th phenotypes, each of them specialized in eliminating different threats. IFN-γ and IL-12 are major drivers of Th1 polarization, whereas IL-4 induces the Th2 program and IL-6 together with TGF-β lead to the Th17 phenotype. Uncontrolled effector T cell responses may result in excessive inflammation and, eventually, in host pathology. In this regard, Th1 and Th17 cells have been associated with various human autoimmune conditions, such as multiple sclerosis (1) and inflammatory bowel disease (2), whereas Th2 cells have been implicated in the development of human allergic asthma (3). Given that CD4$^+$ T cells not only play a key role in protecting the organism from various threats but are also potentially harmful to self-tissues, their function needs to be under stringent control.

Dopamine (DA) is typically recognized for controlling complex processes such as locomotion, cognition, hormone secretion, renal function, and intestinal motility; however, recent evidence points to DA as a key regulator of the immune response involved in sepsis, autoimmune diseases, and neurodegenerative disorders (4–8). DA operates through engagement of five different DA receptors, termed DRD1–DRD5. Although previous reports show expression of all of these receptors on human CD4$^+$ T cells (9), studies analyzing DA receptor expression on various murine CD4$^+$ T cells subsets have shown contrasting results (10, 11). Interestingly, decreased DA levels are associated with inflammatory processes, such as neuroinflammation of the substantia nigra in the brain of Parkinson's disease patients and of the gut mucosa of inflammatory bowel disease patients (4). We have recently shown that genetic deficiency of the highest affinity DA receptor, dopamine receptor D3 (DRD3), on CD4$^+$ T cells attenuates neuroinflammation and subsequent neurodegeneration on a murine model of Parkinson's disease (7). In line with this, pharmacologic stimulation of DRD3 in human T cells favors integrin activation and expression of IFN-γ and TNF-α, whereas it reduces IL-4 and IL-10 production (12–14). However, evidence linking DRD3 signaling with enhanced activation and inflammatory phenotype on CD4$^+$ T cells has been obtained by in vitro studies using human T cells, making it necessary to determine the contribution and relevance of this receptor on CD4$^+$ T cell physiology in vivo.

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Abbreviations used in this article: Alum, aluminum hydroxide; BAL, bronchoalveolar lavage; DA, dopamine; DRD3, dopamine receptor D3; HDM, house dust mite; shDRD3, short hairpin RNA against DRD3; SOCS, suppressor of cytokine signaling; WT, wild-type.

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In this study, by employing a number of experimental paradigms of inflammation, we demonstrate that DRD3 signaling on $CD4^+$ T cells shifts the balance toward inflammatory Th1 and Th17 phenotypes and unveils DRD3 as a key regulator of T cell–mediated immunity.

Materials and Methods

Mice

Wild-type C57BL/6J (WT, *Drd3*$^{+/+}$; $CD45.2^+$) and *Rag1*$^{-/-}$ mice were obtained from The Jackson Laboratory. *Drd3*$^{-/-}$ mice were donated by Dr. Marc Caron (15). Both OT-II and B6.SJL-*Ptprc*^{a} ($CD45.1^+$) were provided by Dr. Maria Rosa Bono. *Drd3*$^{-/-}$ OT-II mice were generated by crossing parental *Drd3*$^{-/-}$ and OT-II mice. Six- to 10-wk-old mice were used in all experiments. Animals were housed and used according to Fundación Ciencia y Vida handling protocols.

Reagents

PerCP– and allophycocyanin–anti-CD4 (GK1.5), PE–anti-CD44 (IM7), allophycocyanin-Cy7–anti-CD62L (MEL-14), FITC– and allophycocyanin–anti-CD25 (PC61), PE-Cy7–anti-IL-7Rα (A7R34), allophycocyanin–anti-CD27 (LG.3A10), allophycocyanin-Cy7–anti-CD45.2 (104), Pacific Blue–anti-CD45.1 (A20), allophycocyanin–anti-IFN-γ (XMG1.2), PE–anti-IL-17A (TC11-18H10.1), allophycocyanin–anti-T-bet (4B10), Alexa Fluor 488–anti-IL-4 (11B11), PE–anti-IL-5 (TRFK5), purified anti-CD3ε (145-2C11), purified anti-CD28 (37.51), purified anti–IFN-γ (AN-18), purified anti–IL-4 (11B11), purified anti–IL-12 (C17.8) and biotin–anti-mouse IgG1 (RMG1-1) Abs, IL-4, TGF-β1, IL-6, IL-1β, IL-12, IL-2, and monensin were purchased from BioLegend. PE-Cy7–anti-Foxp3 (FJK16s), PE-Cy7–anti-IL-13 (eBio13A), and PE-Cy7–anti-IFN-γ (XMG1.2) Abs were obtained from eBioscience. Purified anti–IL-4 (BVD4-1D11), biotin–anti-IL-4 (BVD6-24G2), and streptavidin-HRP were from BD Biosciences. Anti–suppressor of cytokine signaling (SOCS) 3 (H-103), anti-SOCS5 (M-300), and HRP–anti-rabbit IgG Abs were from Santa Cruz Biotechnology. HRP-conjugated anti-mouse IgG was from Rockland Immunochemicals. Anti–β-actin (AC-15), PMA, ionomycin, and grade VI OVA were from Sigma-Aldrich. CFSE and brefeldin A were purchased from Life Technologies. CFA and Imject aluminum hydroxide (Alum) were obtained from Thermo Scientific. The peptide comprising the amino acids 323–339 of OVA was from GenScript (Piscataway, NJ). House dust mite (HDM; *Dermatophagoides pteronyssinus*) extract was from Greer Laboratories. IMDM medium was supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME (Life Technologies).

$CD4^+$ T cell isolation and activation in vitro

Total $CD4^+$ T cells were obtained by negative selection of splenocytes (Miltenyi Biotec). Naive ($CD4^+CD62L^+CD44^-CD25^-$) T cell isolation was achieved by cell sorting using a FACSAria II (BD Biosciences), obtaining purities >98%. All in vitro experiments were performed using complete IMDM medium. To assess proliferation, naive $CD4^+$ T cells were stained with 5 μM CFSE and stimulated for 3 d with 50 ng/well plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28 Abs on flat-bottom 96-well plates (Thermo Scientific). To force differentiation of the Th phenotypes, naive $CD4^+$ T cells were incubated in the conditions indicated above, in addition to a mixture of cytokines and blocking Abs: Th0, 10 ng/ml IL-2, 1 μg/ml anti–IL-4, and 1 μg/ml anti–IFN-γ; Th1, 20 ng/ml IL-12, 10 ng/ml IL-2, and 5 μg/ml anti–IL-4; Th17, 20 ng/ml IL-6, 5 ng/ml TGF-β1, 5 ng/ml IL-1β, 1 μg/ml anti–IL-4, and 1 μg/ml anti–IFN-γ. To drive generation of Th2 cells, total $CD4^+$ T cells were activated in the presence of 10 ng/ml IL-4, 10 μg/ml anti–IFN-γ, and 10 μg/ml anti–IL-12. At different incubation times, cells were assessed for gene and protein expression.

Retroviral transduction of naive $CD4^+$ T cells

For silencing DRD3 expression, we used the retroviral vector pBullet (16), which was provided by Dr. Hinrich Abken. We inserted a region encoding GFP, U6 promoter, short hairpin RNA against DRD3 (shDRD3; 5′-TGCCCTCTCCTCTTTGGTTTCAACACAAC-3′), and H1 promoter into pBullet vector via NcoI and SalI restriction sites (GenScript). pBullet vector drives the expression of the entire construct by the CMV promoter upstream of the NcoI site. This vector was transfected into Phoenix-AMPHO cells, and GFP^+ cells were purified by cell sorting to generate a stable cell line producing shDRD3 retrovirus in the supernatant. Naive $CD4^+$ T cells were activated under Th1-polarizing conditions and infected with retroviral particles at 24 and 48 h of incubation. Infection was carried out by spinoculating cells with retrovirus in RetroNectin-coated plates (Takara Bio, Shiga, Japan). As a nonsilencing control, we transduced $CD4^+$ T cells with a chimeric Ag receptor–expressing retrovirus. This chimeric Ag receptor was specific for recognition of an irrelevant Ag, the carcinoembryonic Ag, and presented an extracellular spacer region (hinge) derived from the IgG1 sequence, which was used to detect transduced cells. At day 5 of culture, cells were restimulated and analyzed for IFN-γ expression in transduced (GFP^+ or $IgG1^+$) $CD4^+$ T cells by flow cytometry.

Flow cytometry

To analyze cytokine production, cells were restimulated with 1 μg/ml ionomycin and 50 ng/ml PMA for 4 h, either in the presence of 5 μg/ml brefeldin A or monensin. For intracellular staining, cells were first stained with a Zombie Aqua fixable viability kit (BioLegend), followed by staining for cell surface markers. Intracellular staining was done with the Foxp3 staining buffer set (eBioscience). Data were collected with a FACSCanto II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Quantitative RT-PCR

Total RNA extracted from cells using the total RNA EZNA kit (Omega Bio-Tek) was DNase digested using the Turbo DNA-free kit (Ambion), and 1 μg RNA was used to synthesize cDNA utilizing Moloney murine leukemia virus reverse transcriptase (Life Technologies). Quantitative gene expression analysis was performed using Brilliant II SYBR Green quantitative PCR master mix (Agilent Technologies). Primers were used at a concentration of 0.5 μM. Expression of target genes was normalized to *Gapdh*. The sequences of the primers used are the following: *Drd3*, sense, 5′-GAACTCCTTAAGCCCCACCAT-3′, antisense, 5′-GAAGGCCCCG-AGCACAAT-3′; *Tbx21*, sense, 5′-CCTGTTGTGGTCCAAGTTCAAC-3′, antisense, 5′-CACAAACATCCTGTAATGGCTTGT-3′; *Gata3*, sense, 5′-GGCGAGATGGTACCGGGCACTA-3′, antisense, 5′-CCCCATTAGC-GTTCCTCCTCCAGA-3′; *Rorc*, sense, 5′-CAGAGGAAGTCAATG-TGGGA-3′, antisense, 5′-GTGGTTGTTGGCATTGTAGG-3′; *Ifng*, sense, 5′-CGGCACAGTCATTGAAAGCCTA-3′, antisense, 5′-GTTGCTGATGG-CCTGATTGTC-3′; *Il4*, sense, 5′-CAACGAAGAACACCACAGAG-3′, antisense, 5′-GGACTTGGACTCATTCATGG-3′; and *Gapdh*, sense, 5′-TCCGTGTTCCTACCCCCAATG-3′, antisense, 5′-GAGTGGGAGTTG-CTGTTGAAG-3′.

Western blot

To analyze the expression of SOCS proteins, total $CD4^+$ T cells were allowed to rest in complete media for 2 h at 37°C to extract proteins either directly or after activation with plate-bound anti-CD3 and soluble anti-CD28 Abs. Samples containing 25 μg protein were run on denaturing conditions and transferred to polyvinylidene difluoride membranes (Thermo Scientific). SOCS3 and SOCS5 proteins were detected with rabbit polyclonal Abs and HRP-conjugated secondary Abs against rabbit IgG. β-Actin expression was detected using mouse mAbs and HRP-conjugated secondary Abs against mouse IgG. Immunodetection was carried out using SuperSignal West Femto chemiluminescent substrate (Thermo Scientific).

Activation and differentiation of $CD4^+$ T cells in vivo

Recipient $CD45.1^+$ mice received 10^5 total or naive $CD45.2^+$ OT-II $CD4^+$ T cells i.v. 1 d before being immunized s.c. with 100 μg OVA peptide in CFA. Ten days later, viable $CD4^+CD45.2^+$ cells obtained from the draining inguinal lymph node were analyzed either directly for the expression of surface markers or after restimulation for cytokine and transcription factor expression.

To assess the generation of Th2 cells in vivo, recipient *Rag1*$^{-/-}$ mice received 10^7 of a mixture of total *Drd3*$^{+/+}$ or *Drd3*$^{-/-}$ OT-II $CD4^+$ T cells and WT $CD4^+$ T cell–depleted splenocytes (in a 15:85 ratio) i.v. 1 d before being immunized i.p. with 20 μg OVA protein in Alum adjuvant. After 14 d, a boost immunization was given, and 4 d afterward mice were processed to obtain spleen and serum. Spleen cells were stimulated for 3 d in the presence of 100 μg/ml OVA protein to analyze IL-4 secretion in supernatants. Serum samples were assessed for the presence of OVA-specific IgG1 Abs by ELISA.

Adoptive transfer chronic colitis model

Recipient *Rag1*$^{-/-}$ mice received 5×10^5 naive ($CD45RB^{high}$) or regulatory/memory ($CD45RB^{low}$) $CD4^+$ T cells i.p. and the body weight of each animal was recorded weekly. After 10 wk, mice were sacrificed to obtain spleen, mesenteric lymph nodes, and colon. The frequency of effector and regulatory $CD4^+$ T cells was assessed after restimulation by flow cytometry. Transverse sections of fixed colon were cut to 5 μm with a

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cryostat, mounted on xylanized slides, and H&E stained to assess intestinal inflammation by light microscopy, as previously described (17).

Allergic asthma model

Mice were sensitized intratracheally with 1 μg HDM extracts dissolved in 80 μl PBS on day 1 and subsequently challenged with five intratracheal doses of 10 μg HDM on days 7–11. Three days after the last challenge, mice were sacrificed and the bronchoalveolar lavage (BAL) was collected to assess cell populations by flow cytometry, as described (18). Polystyrene microspheres (Life Technologies) were included in each sample to determine the cell count of the different populations analyzed.

Statistical analysis

All values are expressed as the mean ± SEM. Statistical analyses were performed with a two-tailed Student t test when comparing only two groups, and with a one-way ANOVA followed by a Tukey post hoc test when comparing more than two groups (GraphPad Software). A p value $<$ 0.05 was considered significant.

Results

TCR signaling induces Drd3 *transcript expression on CD4⁺ T cells in a differentiation-independent manner*

Previous studies regarding DRD3 expression have been focused on human-derived T cells and have shown conflicting results (10, 11, 13). Thus, we analyzed *Drd3* mRNA expression on various $CD4^+$ T cell populations obtained from WT mice. Although *Drd3* transcripts were not detected on naive $CD4^+$ T cells, transient expression of *Drd3* mRNA was induced 2 d after T cell activation driven by CD3 and CD28 cross-linking in the presence of IL-2 (Fig. 1A). Similar results were obtained when naive $CD4^+$ T cells were activated with anti-CD3 and IL-2 mAbs, anti-CD3 mAbs alone, and even when including blocking mAbs to IL-2 (Fig. 1A and data not shown), suggesting that TCR stimulation induces *Drd3* expression on recently activated $CD4^+$ T cells. Because $CD4^+$ T cells are activated in a cytokine-rich environment that dictates lineage specification, we investigated *Drd3* expression under polarizing conditions inducing effector Th1, Th2, and Th17 phenotypes. Interestingly, similar *Drd3* mRNA levels were detected regardless of the effector phenotype acquired by $CD4^+$ T cells (Fig. 1B), suggesting that the different effector $CD4^+$ T cell lineages analyzed might be influenced by DRD3 signaling. Taken together, these data indicate that TCR stimulation induces *Drd3* mRNA expression on $CD4^+$ T cells irrespective of the differentiation program, suggesting that the function of effector phenotypes may be regulated by DRD3 expression.

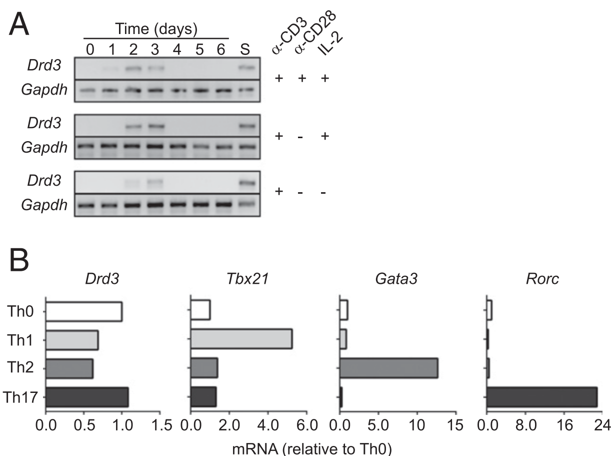

FIGURE 1. TCR signaling induces *Drd3* transcript expression on $CD4^+$ T cells irrespective of the differentiation program. (**A**) Expression of *Drd3* mRNA on naive $CD4^+$ T cells activated under the indicated culture conditions. S, mouse striatum. (**B**) Expression of indicated transcripts on $CD4^+$ T cells after 2 d of activation under neutral (Th0) or skewing conditions (Th1, Th2, Th17). Representative results from five (A) or three (B) independent experiments are shown.

DRD3 expression on CD4⁺ T cells does not alter activation but favors effector phenotype acquisition in vivo

We next evaluated whether DRD3 expression contributes to $CD4^+$ T cell activation and acquisition of effector phenotype by using $CD4^+$ T cells derived from DRD3-deficient ($Drd3^{-/-}$) mice. We have previously shown that activated $Drd3^{-/-}$ $CD4^+$ T cells exhibit a slight impairment in IL-2 secretion compared with their WT counterparts (7, 19). Accordingly, we next tested how DRD3 expression affects $CD4^+$ T cell proliferation. Results showed that proliferative ability of $CD4^+$ T cells was not altered by DRD3 deficiency (Fig. 2A, 2B), indicating that DRD3 expression does not regulate $CD4^+$ T cell activation in vitro.

To test DRD3 contribution to $CD4^+$ T cell activation under physiologic conditions, we transferred $CD4^+$ T cells derived from $Drd3^{-/-}$ mice expressing a transgenic TCR that recognizes a peptide derived from OVA ($Drd3^{-/-}$ OT-II mice) into congenic hosts. Surface expression of CD44, a glycoprotein that promotes extracellular matrix interaction and T cell circulation, was assessed as a T cell activation marker (20). In agreement with in vitro data, CD44 upregulation was similar on both transferred WT and $Drd3^{-/-}$ OT-II $CD4^+$ T cells 10 d after immunization

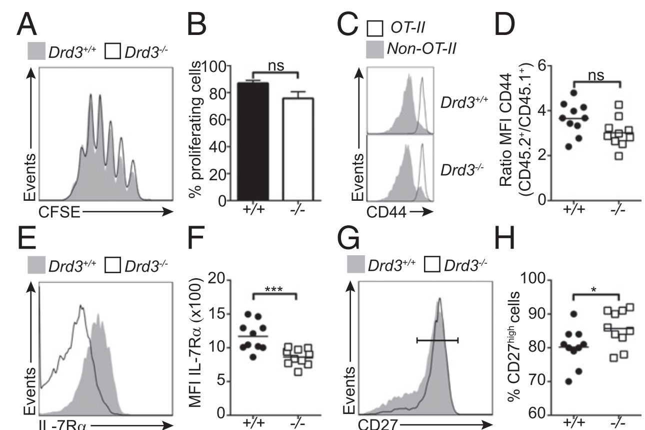

FIGURE 2. DRD3 expression on $CD4^+$ T cells does not alter activation but favors effector phenotype acquisition in vivo. (**A**) Representative proliferation profiles of $Drd3^{+/+}$ (filled histogram) and $Drd3^{-/-}$ (open histogram) naive $CD4^+$ T cells activated for 4 d with anti-CD3 and anti-CD28. (**B**) Percentage of proliferating $Drd3^{+/+}$ (filled column) or $Drd3^{-/-}$ (open column) $CD4^+$ T cells in (A). Data from three independent experiments are shown (n = 3/group). Values represent mean ± SEM. (**C**–**H**) Naive $CD45.2^+$ $Drd3^{+/+}$ or $Drd3^{-/-}$ OT-II cells were i.v. transferred into $CD45.1^+$ recipients. Animals were s.c. immunized with OVA peptide/CFA and inguinal lymph node $CD4^+$ T cells were analyzed 10 d later. (C) Representative histograms showing CD44 expression on transferred $Drd3^{+/+}$ or $Drd3^{-/-}$ (open histograms) OT-II cells. Filled histograms depict CD44 expression on endogenous nontransgenic $CD4^+$ T cells. (D) Ratio of CD44 mean fluorescence intensity among transferred $Drd3^{+/+}$ (●) or $Drd3^{-/-}$ (□) OT-II cells and endogenous non–OT-II cells shown in (C). (E) Representative histograms showing expression of IL-7Rα on transferred $Drd3^{+/+}$ (filled histogram) or $Drd3^{-/-}$ (open histogram) OT-II cells. (F) Mean fluorescence intensity of IL-7Rα staining for donor $Drd3^{+/+}$ (●) or $Drd3^{-/-}$ (□) OT-II cells in (E). (G) Representative histograms showing CD27 expression among transferred $Drd3^{+/+}$ (filled histogram) or $Drd3^{-/-}$ (open histogram) OT-II cells. (H) Frequency of $CD27^{high}$ cells for donor $Drd3^{+/+}$ (●) or $Drd3^{-/-}$ (□) OT-II cells in (G). (D, F, and H) Data from three independent experiments are shown (n = 10/group). Horizontal bars represent mean. $^*p < 0.05$, $^{***}p < 0.001$. MFI, mean fluorescence intensity.

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(Fig. 2C, 2D), suggesting that DRD3 expression is not required for efficient CD4+ T cell activation in vivo. Then, we assessed the expression of surface molecules related to the progression of T cell differentiation into effector phenotype on transferred CD4+ T cells. Expression of IL-7Rα is required for naive T cell survival, but its expression is downregulated upon TCR stimulation (21). Re-expression of this receptor plays a key role in effector and memory cell maintenance, and thereby it reflects the progression of the differentiation process (22, 23). Interestingly, transferred *Drd3*$^{-/-}$ OT-II CD4+ T cells showed significantly lower IL-7Rα expression levels compared with WT OT-II CD4+ T cells 10 d after immunization (Fig. 2E, 2F). Alternatively, the costimulatory molecule CD27 has been shown to be downregulated in terminally differentiated effector cells (24). In agreement with IL-7Rα expression data, the frequency of CD4+ T cells expressing high levels of CD27 was significantly increased among transferred *Drd3*$^{-/-}$ OT-II CD4+ T cells in comparison with WT counterparts (Fig. 2G, 2H). Collectively, these data indicate that whereas DRD3 expression does not participate in CD4+ T cell activation, it contributes to terminal differentiation of effector CD4+ T cells.

Th1, but not Th17, differentiation is favored by DRD3 expression on CD4+ T cells

Previous studies showing that pharmacological stimulation of DRD3 modulates cytokine production by human T cells in vitro (13, 14, 25), together with the results presented above, prompted us to investigate whether DRD3 expression contributes to CD4+ T cell differentiation into effector phenotypes. Whereas *Drd3*$^{-/-}$ CD4+ T cells induced IL-17A expression similarly to their WT counterparts under Th17-skewing conditions, they showed a significantly lower frequency of IFN-γ–producing cells when exposed to Th1-polarizing signals (Fig. 3A, 3B). No IL-4 expression was detected under these conditions either by WT or *Drd3*$^{-/-}$ CD4+ T cells (data not shown). Additionally, among IFN-γ+ cells, CD4+ T cells lacking DRD3 expressed significantly lower amounts of IFN-γ on a per cell basis compared with WT CD4+ T cells (Fig. 3B). In agreement with this, *Drd3*$^{-/-}$ CD4+ T cells exhibited just a slight induction of *Tbx21* and *Ifng* transcripts under Th1 culture conditions (Supplemental Fig. 1A). To rule out the possibility that the observed phenotype results from linkage disequilibrium associated to *Drd3* gene knockout, we silenced DRD3 expression on activated WT CD4+ T cells using retroviral transduction. In line with results shown above, WT CD4+ T cells activated under Th1 conditions and transduced with an shDRD3-expressing retrovirus show a significant reduction in the frequency of IFN-γ–producing cells when compared with CD4+ T cells transduced with a nonsilencing control retrovirus (Supplemental Fig. 1B). Taken together, these results suggest that DRD3 expression contributes to the differentiation of Th1 effector cells, without altering Th17 polarization in vitro.

To validate these results in a physiologic context, we assessed CD4+ T cell differentiation in vivo using the OVA-specific experimental approach described in the previous section (Fig. 2C–H). Differentiation into IL-17A+ cells was similar between normal and *Drd3*$^{-/-}$ CD4+ T cells in vivo (Fig. 3C, 3D). In contrast, the frequency of IFN-γ+ cells was dramatically reduced among transferred *Drd3*$^{-/-}$ OT-II CD4+ T cells when compared with normal OT-II CD4+ T cells (Fig. 3C, 3D). Consistently, DRD3 deficiency resulted in a drastic reduction in the fraction of CD4+ T cells expressing T-bet alone or together with IFN-γ (Fig. 3C, 3D), supporting the notion that DRD3 signaling induces the T-bet/IFN-γ axis on CD4+ T cells, thus favoring Th1, but not Th17, differentiation during the course of an in vivo immune response.

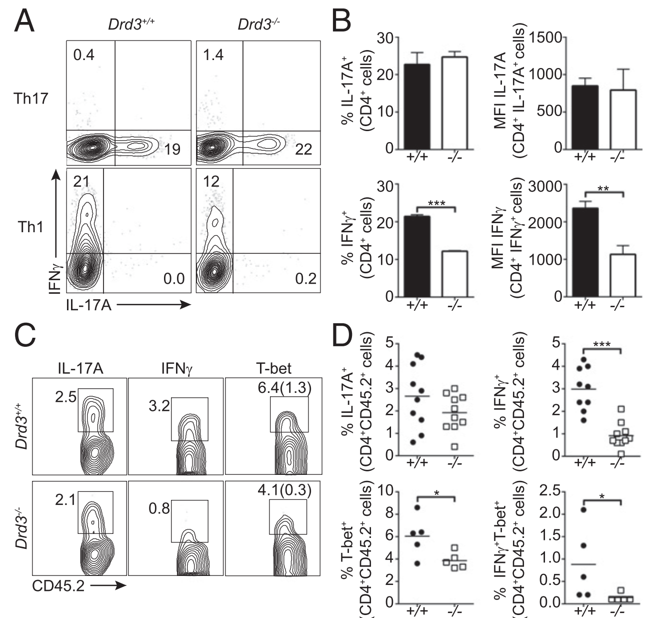

FIGURE 3. DRD3 favors acquisition of Th1 phenotype by CD4+ T cells, without effect in Th17 differentiation. (**A** and **B**) *Drd3*$^{+/+}$ and *Drd3*$^{-/-}$ naive CD4+ T cells were activated in vitro under Th17- and Th1-polarizing conditions for 4 d. (A) Representative plots showing IFN-γ and IL-17A production by CD4+ T cells. Numbers in quadrants indicate the frequency of cytokine-positive CD4+ T cells. (B) Frequency (*left panels*) and mean fluorescence intensity (MFI) (*right panels*) of cytokine-positive *Drd3*$^{+/+}$ (filled column) and *Drd3*$^{-/-}$ (open column) CD4+ T cells. Values represent mean ± SEM. (**C** and **D**) Naive CD45.2+CD4+ *Drd3*$^{+/+}$ or *Drd3*$^{-/-}$ OT-II cells were i.v. transferred into CD45.1+ recipients. Animals were s.c. immunized with OVA peptide/CFA and inguinal lymph node CD4+ T cells were analyzed 10 d later. (C) Representative plots showing IL-17A, IFN-γ, and T-bet expression on transferred *Drd3*$^{+/+}$ or *Drd3*$^{-/-}$ CD4+ OT-II cells. Numbers indicate the percentage of marker-positive CD4+ T cells among transferred cells. Numbers in parenthesis correspond to the frequency of donor CD4+ T cells coexpressing IFN-γ and T-bet. (D) Frequency of marker-positive *Drd3*$^{+/+}$ (●) and *Drd3*$^{-/-}$ (□) CD4+ T cells among transferred cells. (B and D) Data from three independent experiments are shown [n = 3/group in (B); n = 5–10/group in (D)]. Horizontal bars represent mean. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

DRD3 favors inflammatory potential of CD4+ T cells

We next assessed whether impaired differentiation of *Drd3*$^{-/-}$ CD4+ T cells results in reduced inflammatory ability. For this purpose, we used an intestinal inflammation model based on naive CD45RB^{high}CD4+ T cell transfer into lymphopenic hosts (17). Results showed that whereas normal naive CD4+ T cells induced overt weight loss on recipient mice, *Drd3*$^{-/-}$ naive CD4+ T cells caused a significantly milder weight loss on host mice (Fig. 4A), suggesting that DRD3 expression contributes to the inflammatory potential of CD4+ T cells in a colitis model. Histological analysis revealed that *Drd3*$^{-/-}$ naive CD4+ T cells induce mild mucosal inflammation relative to normal naive CD4+ T cell recipients (Fig. 4B). Because the proportion of CD4+ T cells present in the spleen, mesenteric lymph node, and colonic lamina propria was similar in all groups at the end of the experiment (Supplemental Fig. 2 and data not shown), we next analyzed the phenotype of CD4+ T cells. In agreement with the data above (Fig. 3), the proportion of IFN-γ+ cells was markedly reduced on recipients of *Drd3*$^{-/-}$ naive CD4+ T cells when compared with recipients of normal naive CD4+ T cells, resembling what occurs in acute immune responses (Fig. 4C, 4D). Unexpectedly, recipients of DRD3-deficient naive CD4+ T cells showed frequencies of both IL-17A+ as well as IL-17A+IFN-γ+ CD4+ T cells comparable to those of

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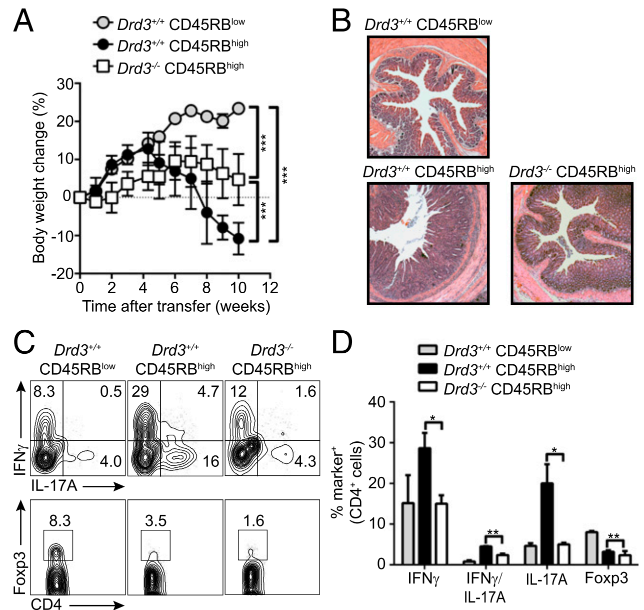

FIGURE 4. DRD3 contributes to the inflammatory potential of $CD4^+$ T cells. (**A**) Body weight change relative to initial weight of *Rag1*$^{-/-}$ recipients of *Drd3*$^{+/+}$ $CD45RB^{low}$ (gray), *Drd3*$^{+/+}$ $CD45RB^{high}$ (black), and *Drd3*$^{-/-}$ $CD45RB^{high}$ (white) $CD4^+$ T cells. (**B**) Representative H&E staining of colon sections from *Rag1*$^{-/-}$ recipients in (A), 10 wk after T cell transfer. Original magnification ×100. (**C**) Representative plots showing the expression of IFN-γ, IL-17A, and Foxp3 for donor $CD4^+$ T cells isolated from the spleen of recipient mice in (A), 10 wk after T cell transfer. (**D**) Frequency of IFN-γ^+, IFN-γ^+/IL-17A$^+$, IL-17A$^+$, and Foxp3$^+$ among donor cells obtained in (C). (A and D) Data from two independent experiments are shown (n = 8–10/group). Values represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

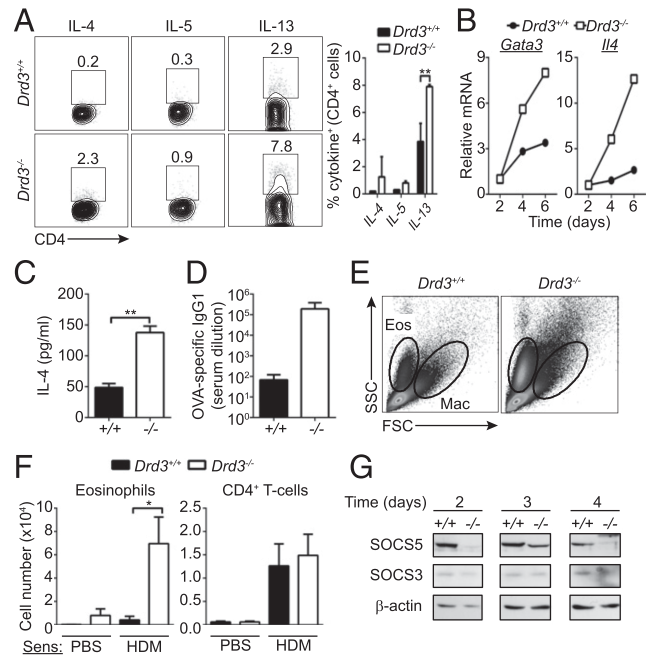

FIGURE 5. DRD3 expressed in $CD4^+$ T cells impairs Th2 differentiation. (**A**) Production of IL-4, IL-5, and IL-13 by *Drd3*$^{+/+}$ and *Drd3*$^{-/-}$ $CD4^+$ T cells activated in vitro under Th2-polarizing conditions for 6 d. Numbers in quadrants indicate the frequency of cytokine-positive $CD4^+$ T cells. (**B**) Expression of *Gata3* and *Il4* transcripts on $CD4^+$ T cells from (A). Results are representative of three independent experiments. (**C**) IL-4 secretion by OVA-stimulated splenocytes obtained from *Rag1*$^{-/-}$ recipients of *Drd3*$^{+/+}$ (filled column) or *Drd3*$^{-/-}$ (open column) OT-II cells immunized with OVA protein/Alum. Both groups of mice also received $CD4^+$-depleted splenocytes from WT mice. (**D**) Titer of OVA-specific IgG1 present in the serum of mice indicated in (C). (**E**) Representative plots showing eosinophil and macrophage gates used to analyze the BAL of *Drd3*$^{+/+}$ and *Drd3*$^{-/-}$ mice sensitized and challenged with the Th2 allergen HDM. (**F**) Eosinophil and $CD4^+$ T cell numbers present in the BAL of *Drd3*$^{+/+}$ (filled column) and *Drd3*$^{-/-}$ (open column) mice challenged with HDM. Sens, sensitized. (**G**) Immunoblot for SOCS5 and SOCS3 expression in *Drd3*$^{+/+}$ or *Drd3*$^{-/-}$ $CD4^+$ T cells cultured in the presence of anti-CD3 and anti-CD28 mAbs for the indicated time periods. Representative results from three independent experiments are shown. (A, C, D, and F) Data from two independent experiments are shown [n = 3/group in (A); n = 6/group in (C), (D), and (F)]. Values represent mean ± SEM. *$p < 0.05$, **$p < 0.01$.

recipients of WT $CD45RB^{low}CD4^+$ T cells (Fig. 4C, 4D), suggesting that DRD3 signaling on $CD4^+$ T cells contributes to both Th1 and Th17 expansion under chronic inflammatory conditions. Finally, the percentage of Foxp3-expressing cells was also significantly reduced on recipients of *Drd3*$^{-/-}$ naive $CD4^+$ T cells (Fig. 4C, 4D), suggesting that DRD3 expression might contribute to regulatory T cell homeostasis and ruling out the possibility that reduced inflammation seen on recipients of DRD3-deficient naive $CD4^+$ T cells is due to excessive expansion of $Foxp3^+$ regulatory T cells. Collectively, these results imply that DRD3 signaling favors the inflammatory abilities of $CD4^+$ T cells during a chronic colitis model.

DRD3 signaling limits Th2 differentiation

Given that Th2-differentiating $CD4^+$ T cells express *Drd3* mRNA (Fig. 1B), and considering the reciprocal regulation of Th1 and Th2 differentiation programs (26), we hypothesized that DRD3 expression on $CD4^+$ T cells may inhibit Th2 phenotype. To address this possibility, we assessed Th2 differentiation of DRD3-sufficient and DRD3-deficient $CD4^+$ T cells both in vitro and in vivo. Whereas WT $CD4^+$ T cells activated under Th2-skewing conditions expressed marginal levels of Th2-related cytokines, DRD3-deficient $CD4^+$ T cells produced significantly more IL-13 than did WT counterparts (Fig. 5A). In agreement, *Drd3*$^{-/-}$ $CD4^+$ T cells cultured under Th2-polarizing conditions transcribed significantly more *Gata3* and *Il4* in comparison with WT $CD4^+$ T cells (Fig. 5B), suggesting that DRD3 signaling on $CD4^+$ T cells restricts Th2 differentiation in vitro.

To verify whether DRD3 expressed on $CD4^+$ T cells also regulates Th2 differentiation in vivo, we transferred *Drd3*$^{+/+}$ or *Drd3*$^{-/-}$ OT-II $CD4^+$ T cells into immune-deficient *Rag1*$^{-/-}$ recipient mice, which were then immunized with OVA protein in Alum adjuvant, a potent inducer of Th2 responses (27). To assess the humoral response, recipients of OT-II cells were cotransferred with $CD4^+$ T cell–depleted splenocytes derived from WT mice. DRD3-deficient OT-II cells responded to OVA protein, secreting significantly higher amounts of IL-4 in comparison with DRD3-sufficient OT-II cells (Fig. 5C). Consistently, recipients of DRD3-deficient OT-II cells presented higher serum titers of OVA-specific IgG1 Abs (Fig. 5D), suggesting that DRD3 expressed on $CD4^+$ T cells hampers Th2 differentiation in vivo. In light of these results, we next assessed whether the increased tendency to differentiate toward Th2 phenotype displayed by DRD3-deficient $CD4^+$ T cells results in higher inflammatory potential in an allergic asthma context (28). BAL obtained from *Drd3*$^{-/-}$ mice displayed significantly higher eosinophil counts in response to a HDM extract when compared with WT mice (Fig. 5E, 5F). Interestingly, this difference cannot be explained by increased $CD4^+$ T cell numbers in the airways of DRD3-deficient mice (Fig. 5F), suggesting that eosinophilia seen on mice lacking DRD3 signaling is likely due to increased Th2 differentiation of $CD4^+$ T cells. Thus,

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these results indicate that Th2 differentiation and function are limited by DRD3 signaling on CD4$^+$ T cells.

To provide a molecular mechanism explaining the regulation of different Th lineages by DRD3 expression on CD4$^+$ T cells, we studied proteins of the SOCS family that have been reported as modulators of CD4$^+$ T cell differentiation. We evaluated the expression of SOCS3, which limits the IL-12–induced STAT4 activation and consequently the Th1 program (29). Additionally, we determined the degree of SOCS5 induction, which attenuates the IL-4–mediated activation of the STAT6/GATA3 axis and thereby the Th2 program (30, 31). Whereas SOCS3 expression was similarly induced on activated CD4$^+$ T cells from both genotypes (Fig. 5G), *Drd3*$^{-/-}$ CD4$^+$ T cells displayed notably reduced SOCS5 levels in response to activation cues when compared with their WT counterparts (Fig. 5G). Altogether, this evidence strongly suggests that DRD3 signaling limits Th2 differentiation by favoring activation-induced SOCS5 expression on CD4$^+$ T cells.

Discussion

This study demonstrates the in vivo relevance of the dopaminergic stimulation of DRD3 expressed on CD4$^+$ T cells and provides a mechanism by which the receptor plays a critical role in defining the balance between effector Th phenotypes. In this regard, by favoring SOCS5 expression and attenuating Th2 differentiation, DRD3 signaling on CD4$^+$ T cells supports the acquisition of the Th1 phenotype. Moreover, under chronic inflammatory conditions, DRD3 favors the expansion of Th17 cells. Thus, the overall effect of DRD3 stimulation in CD4$^+$ T cells is to promote the inflammatory potential of CD4$^+$ T cells.

The dopaminergic regulation of the immune response depends not only on the presence of DA in the particular organ or tissue, but also on the expression of different DA receptors in target immune cells. Our results indicate that Ag recognition by naive CD4$^+$ T cells induces DRD3 expression, rendering this population sensible to this neurotransmitter during the activation/differentiation process. In a physiologic context, DRD3 expressed on recently activated CD4$^+$ T cells may be stimulated by DA released by neighboring dendritic cells present in the lymph node, or by sympathetic dopaminergic terminals reported to innervate both primary and secondary lymphoid organs (32–34). The latter option seems the most likely, considering that we detect DRD3 expression on CD4$^+$ T cells between 2 and 3 d after activation and that studies using intravital microscopy have shown that in vivo–activated CD4$^+$ T cells reduce their interaction with dendritic cells after 40 h of residence in the lymph node (35). Interestingly, retention of activated CD4$^+$ T cells on lymph nodes coincides with the time frame of DRD3 expression found in this work, suggesting that stimulation of DRD3 by dopaminergic sympathetic terminals would be coordinated with the time that recently activated CD4$^+$ T cells spend on lymph nodes. Future work will be required to address whether these events also occur in vivo. It is probable that the transitory induction of *Drd3* mRNA follows the period in which CD4$^+$ T cells are most efficiently stimulated by plate-bound Abs, opening the possibility that CD4$^+$ T cells express DRD3 after the first Ag exposure as well as in subsequent encounters with cognate Ag. DRD3 re-expression on effector CD4$^+$ T cells could have important effects on inflammation sites presenting low concentrations of DA. This is the case in the murine model of Parkinson's disease, in which CD4$^+$ T cells infiltrate the substantia nigra, a brain region with reduced DA levels due to dopaminergic neurodegeneration (36). We showed previously that DRD3 expression on CD4$^+$ T cells is required for the induction of neuroinflammation in this model (7). Thus, it is possible that re-expression of DRD3 on brain-infiltrating CD4$^+$ T cells could enhance the inflammatory profile of these cells and further potentiate neuroinflammation. Based on this evidence, we suggest that reduction on DRD3 levels detected on T cells derived from Parkinson's disease patients corresponds to a compensatory mechanism oriented to reduce the inflammatory potential that low DA levels induce on CD4$^+$ T cells (37). In the case of inflammatory bowel diseases, a reduction of DA levels on inflamed mucosal tissues has been described, suggesting that DRD3-expressing effector CD4$^+$ T cells could respond to low DA levels, contributing to the inflammatory process (38, 39).

Previous studies regarding the expression of DRD3 on CD4$^+$ T cells have shown conflicting results. Watanabe et al. (10) reported that expression of *Drd3* mRNA could not be detected in murine CD4$^+$ T cells, but only in CD8$^+$ T cells. This apparent inconsistency can be explained by the use of a less sensitive detection method together with the analysis of the total population of CD4$^+$ T cells, in which the naive fraction of cells that were found to express very low levels of *Drd3* transcript predominates. Alternatively, Kipnis et al. (11) found that both CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ populations expressed detectable levels of *Drd3* mRNA. The detection of this receptor on CD4$^+$CD25$^-$ T cells could be explained by the expression of DRD3 in the fraction of memory CD4$^+$ T cells contained in the CD4$^+$CD25$^-$ subset. Thus, the present study provides support to previous findings obtained with human samples by analyzing not only naive CD4$^+$ T cells but also effector Th cells.

Although some studies based on pharmacologic stimulation of DA receptors have suggested that DA could favor T cell activation by promoting surface expression of integrins and the secretion of TNF-α (12, 14), it has also been suggested that DA is able to potently inhibit T cell activation, proliferation, and cytokine secretion (40–42). However, most of these studies use mixtures of T cell populations, high DA concentrations, and pharmacologic compounds of limited specificity. In this study, it was possible to discard the contribution of DRD3 in the proliferation and acquisition of an activated phenotype by CD4$^+$ T cells. Regarding the regulation of effector phenotypes, it was previously shown that stimulation of human activated CD4$^+$ T cells with a selective DRD3 agonist results in increased IFN-γ expression along with a reduction in IL-4 and IL-10 production in vitro (13). In line with this evidence, our data not only recapitulate in vivo these results in terms of dual regulation of Th1 and Th2 programs by DRD3 signaling on CD4$^+$ T cells, but they also provide a potential mechanism for this phenomenon. In the present study, we show that DRD3 signaling on activated CD4$^+$ T cells supports SOCS5 expression, a negative regulator of Th2 differentiation that cooperates in stabilizing the Th1 phenotype (31). Our results also agree with previous findings regarding other DA receptors, as Nakano et al. (33) showed that DA dose-dependently promotes Th2 phenotype on human naive CD4$^+$ T cells via D1-like DA receptors. Thus, it is possible to suggest that when DA levels are relatively high, DRD1 and/or DRD5 signaling is dominant over DRD3-triggered effects, thus promoting Th2 differentiation in CD4$^+$ T cells. However, when DA levels are low and DRD3 is selectively stimulated, Th2-promoting signals are lost and Th1 phenotype is favored. The relevance of DRD3 expression on Th1 differentiation was demonstrated using a chronic inflammatory disease model in vivo. Surprisingly, it was shown that DRD3 expression promotes intestinal inflammation driven by CD4$^+$ T cells, by favoring not only Th1, but also Th17 expansion. These results suggest that low DA levels present in the inflamed intestinal mucosa result in promotion of both Th1 and Th17 inflammatory phenotypes by DRD3 signaling on CD4$^+$ T cells.

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Disclosures

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