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Dopamine Receptor D3 Expressed on CD4$^+$ T Cells Favors Neurodegeneration of Dopaminergic Neurons during Parkinson's Disease

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Emerging evidence has demonstrated that CD4$^+$ T cells infiltrate into the substantia nigra (SN) in Parkinson's disease (PD) patients and in animal models of PD. SN-infiltrated CD4$^+$ T cells bearing inflammatory phenotypes promote microglial activation and strongly contribute to neurodegeneration of dopaminergic neurons. Importantly, altered expression of dopamine receptor D3 (D3R) in PBLs from PD patients has been correlated with disease severity. Moreover, pharmacological evidence has suggested that D3R is involved in IFN-γ production by human CD4$^+$ T cells. In this study, we examined the role of D3R expressed on CD4$^+$ T cells in neurodegeneration of dopaminergic neurons in the SN using a mouse model of PD. Our results show that D3R-deficient mice are strongly protected against loss of dopaminergic neurons and microglial activation during 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD. Notably, D3R-deficient mice become susceptible to MPTP-induced neurodegeneration and microglial activation upon transfer of wild-type (WT) CD4$^+$ T cells. Furthermore, RAG1 knockout mice, which are devoid of T cells and are resistant to MPTP-induced neurodegeneration, become susceptible to MPTP-induced loss of dopaminergic neurons when reconstituted with WT CD4$^+$ T cells but not when transferred with D3R-deficient CD4$^+$ T cells. In agreement, experiments analyzing activation and differentiation of CD4$^+$ T cells revealed that D3R favors both T cell activation and acquisition of the Th1 inflammatory phenotype. These findings indicate that D3R expressed on CD4$^+$ T cells plays a fundamental role in the physiopathology of MPTP-induced PD in a mouse model. ***The Journal of Immunology*****, 2013, 190: 000–000.**

Parkinson's disease (PD) is a neurodegenerative disorder, characterized by a pervasive dysfunction and degeneration of midbrain dopaminergic (DAergic) neurons in the substantia nigra (SN) (1). Several lines of evidence suggest that neuroinflammation is crucial in the cascade of events leading to neuronal loss and progression of PD. PD-associated neuroinflammation comprises microglial activation and subsequent T cell infiltration in the SN (2). In this regard, infiltrating T cells have been detected in the SN from PD patients as well as from mice undergoing PD after the treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (3, 4). Importantly, recent studies using TCR-β-chain–deficient mice, SCID mice, and RAG1 knockout (RAG1KO) mice have demonstrated that T cell deficiency results in a strong attenuation of DAergic neurodegeneration in MPTP-induced PD (5, 6), revealing a relevant role for T cells in PD. Additional experiments have shown that whereas CD8$^+$ T cell deficiency is negligible, participation of CD4$^+$ T cells is fundamental for promoting neurodegeneration of DAergic neurons in the SN of mice undergoing PD (6).

Because of the pivotal participation of CD4$^+$ T cells in the development of PD, some studies have attempted to analyze T cell Ags involved in this disorder. In this regard, nitrated α-synuclein, a major component of the Lewy bodies observed on PD, has been suggested as one of the neoantigens recognized by the adaptive immune system during PD (5, 7, 8). Importantly, nitrated α-synuclein is not only present in mice undergoing PD but also in brains of PD patients (5, 9). These studies have shown that nitrated α-synuclein is presented in cervical lymph nodes, which drain Ags from CNS, and elicits an Ag-specific CD4$^+$ T cell response mediated by cells with the inflammatory phenotypes Th1 and Th17, which exacerbate the microglial activation and neuronal damage (5, 8). In contrast, it has been demonstrated that CD4$^+$ regulatory T cells (Tregs), which suppress function of inflammatory Th1 and Th17 cells, may also participate during PD, attenuating the destruction of DAergic neurons in the SN (7, 10). Thus, this evidence supports the participation of a CD4$^+$ T cell response during PD, which seems to be fundamental for the degeneration of DAergic neurons in the SN.

During the past two decades, several studies have shown the capability of several neurotransmitters to regulate the function and differentiation of immune cells, including acetyl-choline, serotonin, glutamate, and dopamine (DA) among others (11–18). Regarding expression of DA receptors (DARs) in immune cells,

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Abbreviations used in this article: DA, dopamine; DAergic, dopaminergic; DAR, DA receptor; DAT, DA transporter; DC, dendritic cell; D3R, DA receptor D3; D3RKO, D3R knockout; FJ-C, Fluoro-Jade C; MAC1, macrophage 1 Ag; MPP$^+$, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; RAG1KO, RAG1 knockout; SN, substantia nigra; SNpc, SN pars compacta; TH, tyrosine hydroxylase; Treg, regulatory T cell; WT, wild-type.

these receptors have been found not only in cells of the innate immune response such as dendritic cells (DCs), NK cells, macrophages/monocytes and granulocytes (19–21) but also in cells of the adaptive immune response such as B cells, $CD8^+$ T cells, and $CD4^+$ T cells (22–30). Despite being lesser studied than in human T cells, DARs expression has also been described in murine T cells (26, 31). Pharmacological evidence obtained from a group of studies performed with human T cells has suggested that among five DARs described so far (D1R–D5R), both type I (D1R and D5R) and type II (D2R, D3R, and D4R) contribute to the regulation of T cell function. In this regard, Nakano et al. (27) have suggested that stimulation of type I DARs expressed on human naive $CD4^+$ T cells would contribute to production of Th2 cytokines, whereas Cosentino et al. (32) have suggested that stimulation of type I DARs on Tregs would decrease IL-10 and TGF-β production. Sarkar et al. (25) have described that D4R stimulation on human T cells promotes quiescence. In contrast, Besser et al. (22) have shown evidence suggesting that stimulation of D2R and D3R in human resting T cells obtained from healthy donors would favor production of IL-10 and TNF-α, respectively. It has also been described that stimulation of D3R in resting T cells favors activation of β_1 integrins and adhesion to fibronectin, two critical events required for cell migration (26, 29). Importantly, Ilani et al. (33) have suggested that D3R stimulation in human activated $CD4^+$ T cells decrease IL-4 and IL-10 synthesis and potentiates IFN-γ production, the hallmark cytokine of Th1 cells. The same authors have shown that pharmacologic D3R stimulation in human T cells potentiate expression of surface activation markers (33). In contrast, Saha et al. (23, 24) have shown that DA, at concentrations that should selectively stimulate D3R, inhibits human T cell proliferation.

Because specific loss of DAergic neurons occurs in PD, DA is the main neurotransmitter affected in this disorder. Thus, it is likely that DARs expressed on brain-infiltrating $CD4^+$ T cells play an important role in the immune response involved in PD. Interestingly, expression of D3R is significantly reduced on circulating lymphocytes obtained from PD patients, and this reduction correlates with disease severity (34), thus suggesting a role for D3R expression on lymphocytes in PD physiopathology. Moreover, pharmacologic evidence has shown that stimulation of D3R expressed on human $CD4^+$ T cells promotes enhanced production of IFN-γ and TNF-α, both of which play critical roles stimulating and maintaining glial cell activation in PD (22, 33, 35).

In this study, we investigated the relevance of D3R expressed on $CD4^+$ T cells in the activation of microglia and degeneration of DAergic neurons in the SN during MPTP-induced PD. For this purpose, we used D3R knockout (D3RKO) mice and also mice lacking D3R specifically in $CD4^+$ T cells or D3RKO mice in which wild-type (WT) $CD4^+$ T cells were transferred. Our results indicate a fundamental role of D3R expressed in $CD4^+$ T cells in the destruction of DAergic neurons during PD in a mouse model.

Materials and Methods

Animals and MPTP intoxication

Ten- to 12-wk-old male mice of the C57BL/6 background were used for all experiments. WT and RAG1 knockout (RAG1KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). D3RKO mice were donated by Dr. M. Caron (Duke University Medical Center, Durham, NC) (36). Mice were injected i.p. with four separate MPTP-HCl doses (4 × 20 mg MPTP/kg; Sigma-Aldrich, St. Louis, MO). Each injection was given at 2 h intervals, and mice were sacrificed 7 d after injections. Control mice received injections containing saline solution. All animal procedures were in accordance to institutional guidelines for the care and use of animals at the pathogen-free animal facility of Fundación Ciencia y Vida.

Tissue preparation and immunohistochemistry

Mice were transcardially perfused with 0.9% NaCl, followed by 4% paraformaldehyde (Sigma-Aldrich). Frozen midbrain sections (20 μm) were immunostained for tyrosine hydroxylase (TH) (anti-TH Ab; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) (37). Fluoro-Jade C (FJ-C) staining was performed on adjacent sections, according to the manufacturer's protocol, to assess degenerating neurons (Millipore, Billerica, MA). Adjacent midbrain sections were immunostained for macrophage 1 Ag (MAC1; CD11b, 1:1000) and CD4 (1:100) both from Serotec (Raleigh, NC). Sections were incubated in HRP-conjugated streptavidin solution (ABC Elite vector kit; Vector Laboratories). The mean number of TH^+ neurons from four SN pars compacta (SNpc) sections per mouse was counted under light microscopy at a magnification of ×200, and the total area of SNpc was calculated using ImageJ software (National Institutes of Health, Bethesda, MD). Density of DAergic neurons was expressed as the number of TH^+ neurons per square millimeter in the SNpc. The mean number of $MAC1^{high}$ reactive microglia displaying ameboid shape and $CD4^+$ cells were counted in areas of interest of 150 × 150 μm^2 in three SN sections per mouse.

Adoptive transfer

Splenocytes obtained from WT or D3RKO mice were transferred i.v. (2×10^7 cells/mouse) into RAG1KO-recipient mice 21 d before MPTP intoxication (Fig. 3A). Successful transfer of splenocytes was confirmed by FACS analysis. There were no differences in percentages of blood $CD4^+$ or $CD8^+$ T cells between RAG1KO mice reconstituted with WT or D3RKO splenocytes, and they were similar to those observed in WT mice. In experiments from Fig. 3B, 21 d before MPTP intoxication, RAG1KO mice were i.v. injected with $CD4^+$ T cell–depleted WT splenocytes (1×10^7 cells/mouse) mixed with WT or D3RKO $CD4^+$ T cells (3×10^6 cells/mouse). There were no differences in percentages of $CD4^+$ and $CD8^+$ T cells from both groups of experimental mice. When D3RKO mice were used as recipient mice (Fig. 4), isolated WT $CD4^+$ T cells were injected i.v. (12×10^6 cells/mouse) 1 d before MPTP treatment. Purification of $CD4^+$ T cells from total splenocytes was carried out by negative selection using a $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), and purity was assessed by FACS (>95% purity).

T cell activation assays

Purification of $CD4^+$ T cells from total splenocytes was carried out by negative selection using $CD4^+$ T cell isolation kits (Miltenyi Biotec). Cells were incubated (2×10^5 cells/well) in plate-bound anti-CD3 and anti-CD28 mAbs (100 ng/well) for 24 h. T cell activation was determined as IL-2 secretion in the culture supernatant by ELISA, as described previously (21).

Analysis of $CD4^+$ T cell differentiation in vitro

Naive $CD4^+CD25^-$ T cells were obtained at >98% purity by cell sorting on a FACSAria II (BD Biosciences, San Jose, CA), and splenic DCs were purified using a $CD11c^+$ magnetic selection kit (Miltenyi Biotec). Cells were cultured on a 5:1 (T:DC) ratio on U-bottom 96-well plates in the presence of 1 μg/ml soluble anti-CD3ε mAb and a mixture of cytokines and neutralizing mAbs to direct differentiation toward Th1, Th17, and Treg phenotypes. Th1 conditions included 5 ng/ml IL-12 and 5 μg/ml anti–IL-4 mAb, whereas Th17 conditions contained 10 ng/ml IL-6, 2.5 ng/ml TGF-β, 5 ng/ml IL-1β, 5 μg/ml anti–IL-4 mAb, and 5 μg/ml anti–IFN-γ mAb. In both conditions, IL-2 (10 ng/ml) was added after 3 d to expand T cells. On day 7, cells were stimulated with 50 ng/ml PMA and 1 μg/ml ionomycin in the presence of 5 μg/ml brefeldin A for 4 h. To assess intracellular cytokine production, cells were stained with PerCP-conjugated anti-CD4 mAb, fixed with 1% formaldehyde, permeabilized with permeabilizing solution (3% BSA and 0.5% saponin in PBS), and then stained with PE-conjugated anti–IL-17 and allophycocyanin-conjugated anti–IFN-γ mAbs. Treg conditions included 10 ng/ml IL-2 and 5 ng/ml TGF-β. After 6 d, cells were stained with PerCP-conjugated anti-CD4 and FITC-conjugated anti-CD25 mAbs, fixed and permeabilized using Foxp3 staining kit (eBioscience, San Diego, CA), and then stained with allophycocyanin-conjugated anti-Foxp3 mAb. Cytokine production and Foxp3 expression were analyzed by flow cytometry on a FACSCanto II (BD Biosciences). All cytokines and mAbs were purchased from BD Biosciences, except anti-Foxp3 mAb (eBioscience). In some experiments in which $CD4^+$ T cells were treated with a selective D3R agonist (PD128907; from Tocris, Bristol, U.K.), a combination of plate-bound anti-CD3 (100 ng/well) and soluble anti-CD28 (2 μg/ml) mAbs was used instead of DCs to avoid the possible effect of the D3R agonist on DC behavior.

Analysis of the functional phenotype of T cells infiltrating the CNS in vivo

Mice were treated with MPTP or saline solution as described above and sacrificed after 5 d. For the preparation of SN-infiltrating mononuclear cells, mice were perfused through the left cardiac ventricle with cold PBS. The SN was dissected from the brain, and then it was cut into small pieces and digested by collagenase D (2.5 mg/ml; Roche Diagnostics) at 37˚C for 45 min. Digested tissue was passed through a 70 μm cell strainer, and the obtained single-cell suspension was subjected to centrifugation in a Percoll gradient (70/37%). Mononuclear cells were removed from the interphase and resuspended in culture medium. Cells were stimulated with 50 ng/ml PMA and 1 μg/ml ionomycin in the presence of 5 μg/ml brefeldin A for 4 h. To assess IFN-γ, TNF-α, and Foxp3 expression in T cells, mononuclear cells were stained with allophycocyanin–cyanine 7-conjugated anti-CD3 mAb, fixed with 1% formaldehyde, permeabilized with permeabilizing solution (3% BSA and 0.5% saponin in PBS), and then stained with allophycocyanin-conjugated anti–IFN-γ, PE–cyanine 7-conjugated anti–TNF-α, and PE-conjugated anti-Foxp3 mAbs. Cytokine production and Foxp3 expression were analyzed by flow cytometry on a FACSCanto II.

Statistical analysis

All values are expressed as mean ± SD. Differences in means between two groups were analyzed using two-tailed Student *t* test by using GraphPad Prism software. Unpaired test was used, except when indicated. Comparisons between data from different treatments but same genotype (or same cell transference) or between data from different genotype (or different cell transference) but same treatments were analyzed in results shown. A p value <0.05 was considered as significant.

Results

D3R deficiency protects from neurodegeneration of DAergic neurons in the SN

To test the global relevance of D3R in the development of PD, we first aimed to determine how D3R deficiency affects disease manifestation. Accordingly, susceptibility of WT and D3RKO mice to MPTP-induced loss of DAergic neurons and general neurodegeneration were evaluated. For this purpose, mice were treated with an acute toxicity regimen of MPTP, and after 7 d, histological analysis of DAergic neuron integrity and neurodegeneration in the SN were performed in midbrain sections by TH immunostaining and FJ-C labeling, respectively. Consistent with previous reports (6, 7, 10), results show that MPTP treatment of WT mice results in significant loss of DAergic neurons (≈40%) in the SNpc. Strikingly, D3R deficiency completely abolished MPTP-induced loss of DAergic neurons (Fig. 1A). TH-immunostaining analysis and subsequent quantification of midbrain sections obtained from PBS-treated (saline) WT and D3RKO mice indicates similar numbers of DAergic neurons in the SNpc of healthy mice from both genotypes (Fig. 1A). To rule out the possibility that, despite still expressing TH, DAergic neurons were degenerating, FJ-C histochemistry was performed to reveal dead or dying neurons. In agreement with MPTP-induced DAergic neuron destruction in WT mice, FJ-C staining reveals prominent neuron death in the SNpc of these mice. In contrast, FJ-C staining in the SNpc from MPTP-treated D3RKO mice was virtually absent and similar to that observed in saline-treated WT or D3RKO mice (Fig. 1B). Thus, these results indicate that D3RKO mice display a robust neuroprotection after acute MPTP treatment.

D3R deficiency attenuates microglial activation but not CD4$^+$ T cell infiltration into the SN

A number of studies have demonstrated the relevance of microglial activation in neuroinflammation and neurodegeneration and the involvement of CNS-infiltrating CD4$^+$ T cells in the regulation of microglia functionality in the scenario of PD and other neurodegenerative diseases (6–8, 10, 38, 39). Accordingly, we investigated microglial activation and CD4$^+$ T cell infiltration into the SN by immunostaining analysis for MAC1 and CD4, respectively, in midbrain sections obtained from MPTP-treated WT and D3RKO mice. Results show an increased expression of the activation marker MAC1 and acquisition of the ameboid morphology characteristic of reactive microglia in the SN of MPTP-treated WT mice (Fig. 2A). In contrast, microglial cells from the SN of MPTP-treated D3RKO mice exhibited only marginal MAC1 expression and retained an elongated and highly ramified morphology that resembles homeostatic microglia (Fig. 2A). Microglial cells from SN obtained from saline-treated WT or D3RKO mice displayed a homeostatic phenotype with low MAC1 expression and elongated and highly ramified bodies (Fig. 2A). Consistent with the crucial role of SN-infiltrating CD4$^+$ T cells in promoting loss of DAergic neurons in MPTP-treated mice (5–7, 10), our results show a significant infiltration of CD4$^+$ T cells into the SN obtained from MPTP-treated WT mice when compared with those SN obtained from saline-treated WT mice (Fig. 2B). Although MPTP-treated D3RKO mice show reduced CD4$^+$ T cell infiltration into the SN, no significant difference was observed when compared with midbrain sections obtained from MPTP-treated WT mice (Fig. 2B). Thus, these data suggest that D3R deficiency attenuates microglial activation without affecting CD4$^+$ T cell infiltration into the SN during MPTP-induced PD in mouse.

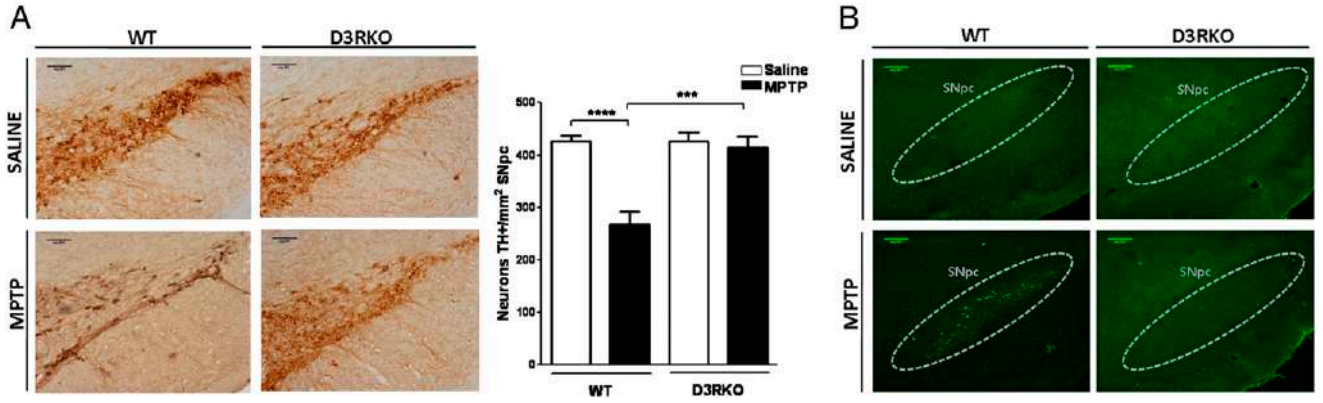

FIGURE 1. D3R deficiency protects from MPTP-induced degeneration of DAergic neurons in the SN. WT or D3RKO mice were i.p. injected with either PBS (saline) or MPTP. After 7 d, loss of DAergic neurons and general neurodegeneration were evaluated in the SNpc by immunohistochemical analysis of TH (**A**) and FJ-C (**B**), respectively. Representative images are shown [scale bars, 100 μm, *left panel* in (A) and (B)]. Quantification of TH$^+$ cells is depicted [(A), *right panel*]. For better understanding images in (B), location of SNpc has been marked by white dotted lines. Data from five independent experiments, including at least 12 mice/group, are shown. ***$p < 0.001$, ****$p < 0.0001$.

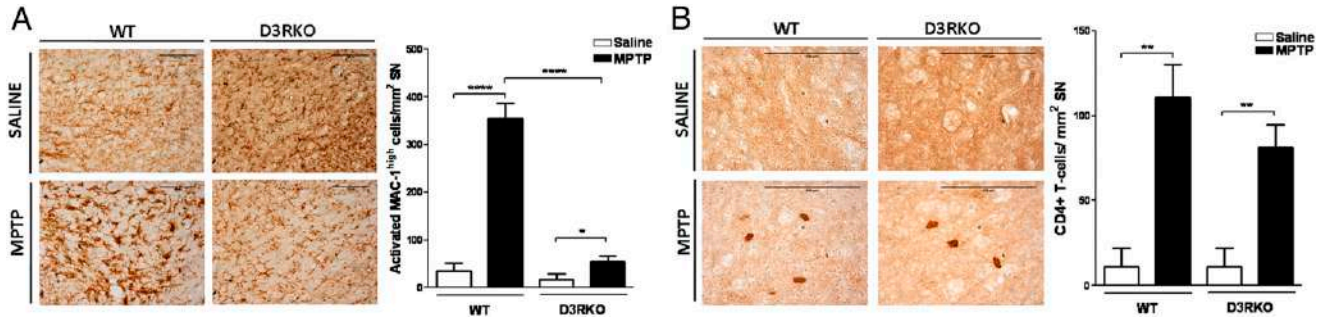

FIGURE 2. D3R deficiency attenuates microglial activation but not CD4+ T cells infiltration into the SN. WT or D3RKO mice were treated as described in Fig. 1 legend. Microglial activation (**A**) and CD4+ T cells infiltration (**B**) were determined in the SN by immunohistochemical analysis of MAC1 and CD4, respectively. Representative images are shown in *left panels* (scale bars, 100 μm) and quantification in *right panels* (A, B). Data from three independent experiments, including at least five mice/group, are shown. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

D3R expressed on CD4+ T cells plays a critical role in the MPTP-induced destruction of DAergic neurons in the SN

Because CD4+ T cells play a key role in the acquisition of microglial phenotype (8, 10, 38, 39), and D3R has been involved in the function of CD4+ T cells (33), we next addressed the question of whether D3R expressed on T cells is relevant for destruction of DAergic neurons in MPTP-induced PD. For this purpose, RAG1KO mice, which are devoid of T and B cells, were used as recipient mice. As a first approximation to analyze the role of D3R expressed on immune cells in the destruction of DAergic neurons in the SN, we transferred WT or D3RKO splenocytes into RAG1KO mice, and 3 wk later, the mice were intoxicated with MPTP. DAergic neuron loss in the SN was analyzed in midbrain sections at day 7 postintoxication. In agreement with previous reports in which T cell–deficient mice are protected against MPTP-induced destruction of DAergic neurons in the SN (5, 6), our results show that MPTP intoxication had no significant effect on the number of TH+ neurons in the SNpc of RAG1KO mice (Supplemental Fig. 1). Importantly, when RAG1KO mice were reconstituted with WT splenocytes, MPTP-treatment promoted a strong loss of DAergic neurons in the SNpc (Fig. 3A), indicating that a cell population present in splenocytes is essential to induce destruction of TH+ neurons. Strikingly, RAG1KO mice reconstituted with D3R-deficient splenocytes were completely resistant against MPTP-induced loss of TH+ neurons in the SNpc (Fig. 3A), indicating that D3R expressed in splenocytes plays a key role in the mechanism of MPTP-evoked destruction of DAergic neurons. Because CD4+ T cells are required for MPTP-mediated loss of TH+ neurons (5, 6), we next sought to determine whether D3R expressed specifically on CD4+ T cells is relevant in the destruction of DAergic neurons during PD. To answer this question, we carried out experiments in which MPTP-induced loss of DAergic neurons was evaluated in the SN of RAG1KO mice reconstituted with a mixture of cells composed of CD4+ T cell–depleted WT splenocytes and CD4+ T cells obtained from WT or D3RKO mice. Results show that, when compared with non-transferred RAG1KO mice (Supplemental Fig. 1), mice bearing WT CD4+ T cells are susceptible to MPTP-induced destruction of DAergic neurons in the SNpc (Fig. 3B). Conversely, MPTP treatment of mice bearing D3R-deficient CD4+ T cells results in negligible destruction of TH+ cells in the SNpc (Fig. 3B). To answer the question of whether D3R expressed specifically on CD4+ T cells was relevant for microglial activation in the SN during MPTP-induced PD, we also analyzed morphology and MAC1 expression in midbrain sections from the same mice. In correlation with the destruction of TH+ neurons, significantly stronger microgliosis was observed in mice bearing WT CD4+ T cells when compared with those mice bearing D3RKO CD4+ T cells (Supplemental Fig. 2). Thus, these results support that D3R expressed on CD4+ T cells is important in promoting microglial activation and neurodegeneration of TH+ neurons in the SN during MPTP-induced PD in mouse. According to this notion and to corroborate these data, we reasoned that transfer of WT CD4+ T cells into D3RKO-recipient mice should make these mice responsive to MPTP-induced destruction of TH+ neurons in the SN. Accordingly, we evaluated loss of DAergic neurons in the SNpc obtained from MPTP-treated WT and D3RKO mice and D3RKO mice in which WT CD4+ T cells were previously transferred. Similar to what we observed before (Fig. 1A), D3RKO mice are refractory to MPTP-induced PD (Fig. 4). However, transfer of WT

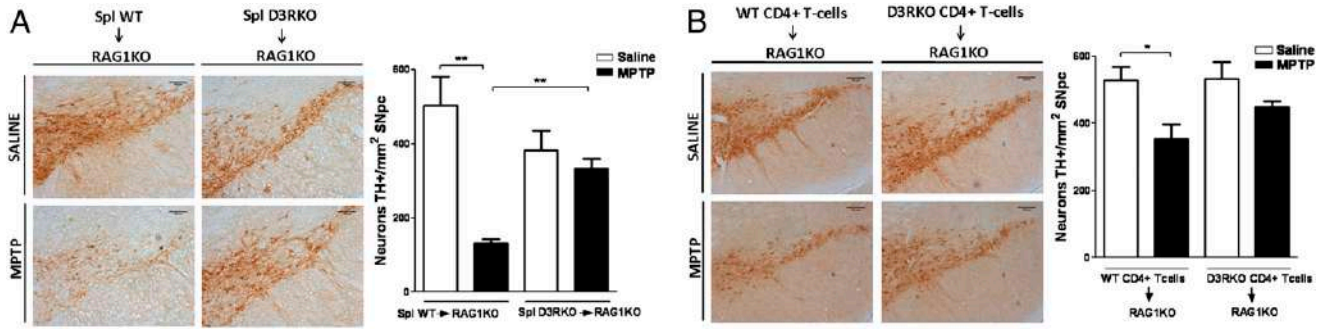

FIGURE 3. CD4+ T cell–specific ablation of D3R attenuates MPTP-induced degeneration of DAergic neurons in the SN. RAG1KO mice were reconstituted with WT or D3RKO splenocytes (**A**) or with CD4+ T cell–depleted WT splenocytes mixed with WT or D3RKO CD4+ T cells (**B**). Twenty-one days after cell transfer, mice were treated with either saline or MPTP, and midbrain sections were analyzed 7 d later. The loss of DAergic neurons was evaluated in the SN by immunohistochemical analysis of TH. Representative images are shown in *left panels* (scale bars, 100 μm) and quantification in *right panels* (A, B). Data from three independent experiments, including four to five mice per group, are shown. $*p < 0.05$, $**p < 0.01$.

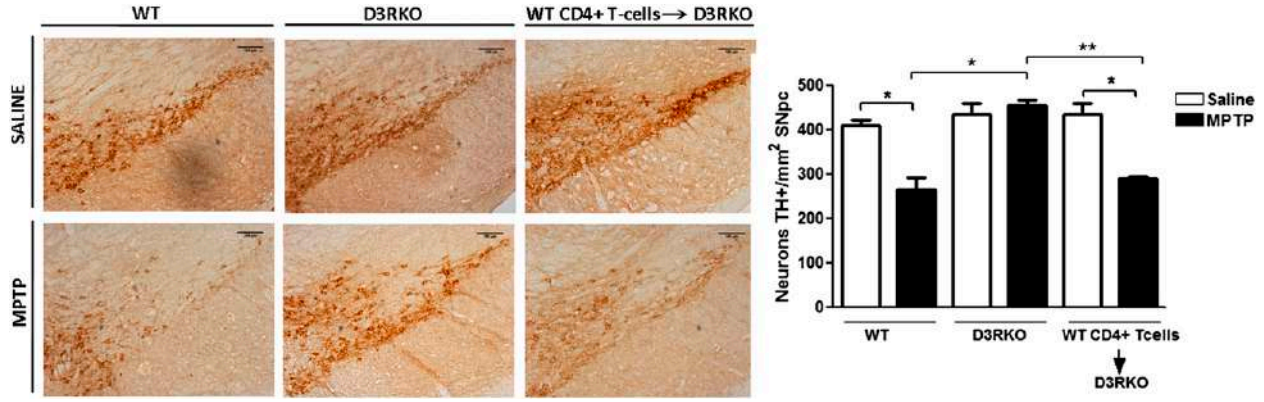

FIGURE 4. $CD4^+$ T cells require D3R expression to promote MPTP-induced degeneration of DAergic neurons in the SN. WT $CD4^+$ T cells were transferred into D3RKO-recipient mice and then treated with either saline or MPTP. Nontransferred WT and D3RKO mice treated with either saline or MPTP were used as controls. The loss of DAergic neurons was evaluated in the SN by imunohistochemical analysis of TH. Representative images are shown in the *left panels* (scale bars, 100 μm) and their quantification in *right panels*. Data from three independent experiments, including four to five mice per group, are shown. $*p < 0.05$, $**p < 0.01$.

$CD4^+$ T cells turns D3RKO mice highly susceptible to MPTP-induction of DAergic neuron loss (Fig. 4). To corroborate the role of D3R expressed on $CD4^+$ T cells in MPTP-induced microgliosis, we also analyzed MAC1 expression in midbrain sections from these mice. Again, microglia activation was only observed in mice bearing WT $CD4^+$ T cells but not in mice lacking D3R in $CD4^+$ T cells (Supplemental Fig. 3). Hence, this group of results indicates that D3R expressed on $CD4^+$ T cells is essential to promote MPTP-mediated microglial activation and destruction of DAergic neurons.

D3R expressed on $CD4^+$ T cells favors T cell activation and acquisition of the proinflammatory Th1 phenotype

Pharmacological evidence has shown that D3R stimulation of human T cells in vitro may modulate both expression of T cell activation markers and production of effector cytokines (22–24, 33). Accordingly, we next addressed the question of whether D3R expressed on $CD4^+$ T cells affects T cell activation and differentiation. For this purpose, we first performed T cell activation assays to determine the role of D3R in the production of IL-2 using both genetic and pharmacologic approaches. Results show that lack of D3R in $CD4^+$ T cells results in an impaired T cell activation (Fig. 5A). Moreover, stimulation of WT $CD4^+$ T cells with a specific D3R agonist (PD128907) potentiates IL-2 production (Fig. 5B). Thus, these results together indicate that D3R expressed in $CD4^+$ T cells plays an important role favoring T cell activation. Previous studies addressing the role of T cells in PD have shown that DAergic neuronal loss in the SN is associated to proinflammatory phenotypes of $CD4^+$ T cells, such as Th1 and Th17. Conversely, participation of $CD4^+$ T cells with the anti-inflammatory phenotype Tregs has been associated to attenuated MPTP-induced loss of TH^+ neurons in the SN (7, 10, 38). Accordingly, we next performed experiments to determine the involvement of D3R expressed on $CD4^+$ T cells in the acquisition of proinflammatory and anti-inflammatoty phenotypes described to be relevant for PD. For this purpose, naive $CD4^+CD25^-$ T cells were isolated from WT and D3RKO mice and cocultured with WT DCs in conditions favoring acquisition of Th1, Th17, or Treg phenotypes. Importantly, in this experimental approach, WT DCs not only participate as APCs for T cell activation and differentiation but also they constitute a source of DA for stimulation of DARs in the cell culture (21). Results show that when compared with WT $CD4^+$ T cells, D3R deficiency impairs significantly the capability of these cells to produce IFN-γ when cultured under Th1 conditions (Fig. 5C). In contrast, $CD4^+$ T cells lacking D3R displayed similar capability to acquire Th17 or Treg phenotypes when compared with WT $CD4^+$ T cells (Fig. 5C). To complement this genetic evidence about the role of D3R expressed on $CD4^+$ T cells in the acquisition of the Th1 phenotype, we next performed in vitro differentiation experiments in which WT naive $CD4^+$ T cells were treated with a specific D3R agonist, and the effector phenotype acquired was evaluated. Because D3R has also been described in DCs (19, 21), we used anti-CD3 and anti-CD28 mAbs instead of DCs in these experiments to avoid effects as a result of the stimulation of D3R expressed on DCs. In agreement with genetic evidence (Fig. 5C), these results show that D3R stimulation favors acquisition of the Th1 phenotype (Fig. 5D). Thus, these results together indicate that D3R expressed on $CD4^+$ T cells play an important role in the differentiation of naive $CD4^+$ T cells toward the Th1 phenotype. Finally, to address the relevance that D3R plays in vivo in the functional phenotype of T cells infiltrated in the SN of MPTP-treated mice, we analyzed the production of two important inflammatory cytokines, IFN-γ and TNF-α, and expression of Foxp3 by these cells in WT and D3RKO mice undergoing PD. Results show a strong induction of IFN-γ and TNF-α production by T cells infiltrating the SN in WT mice treated with MPTP. In striking contrast, this induction is absent in D3R-deficient mice (Fig. 5E). In addition, our results show an increase in the frequency of $Foxp3^+$ T cells infiltrating the SN when D3RKO mice were treated with MPTP, an effect absent in MPTP-treated WT mice (Fig. 5E). Taken together, this group of results suggests that D3R expressed on $CD4^+$ T cells contributes both to T cell activation and to the acquisition of an inflammatory T cell phenotype, which consequently favors neurodegeneration in mice undergoing MPTP-induced PD.

Discussion

In this work, we present data from in vivo experiments demonstrating that D3R-deficient mice are resistant to microglial activation and degeneration of DAergic neurons in the SN when PD is induced. Moreover, when WT $CD4^+$ T cells were transferred into D3R-deficient mice, these animals became susceptible to neurodegeneration upon PD induction. Furthermore, in experiments using mice lacking D3R specifically in $CD4^+$ T cells, we demonstrated that D3R expressed in $CD4^+$ T cells is fundamental to promote destruction of DAergic neurons and microgliosis upon PD. Mechanistic analysis suggests an involvement of D3R in

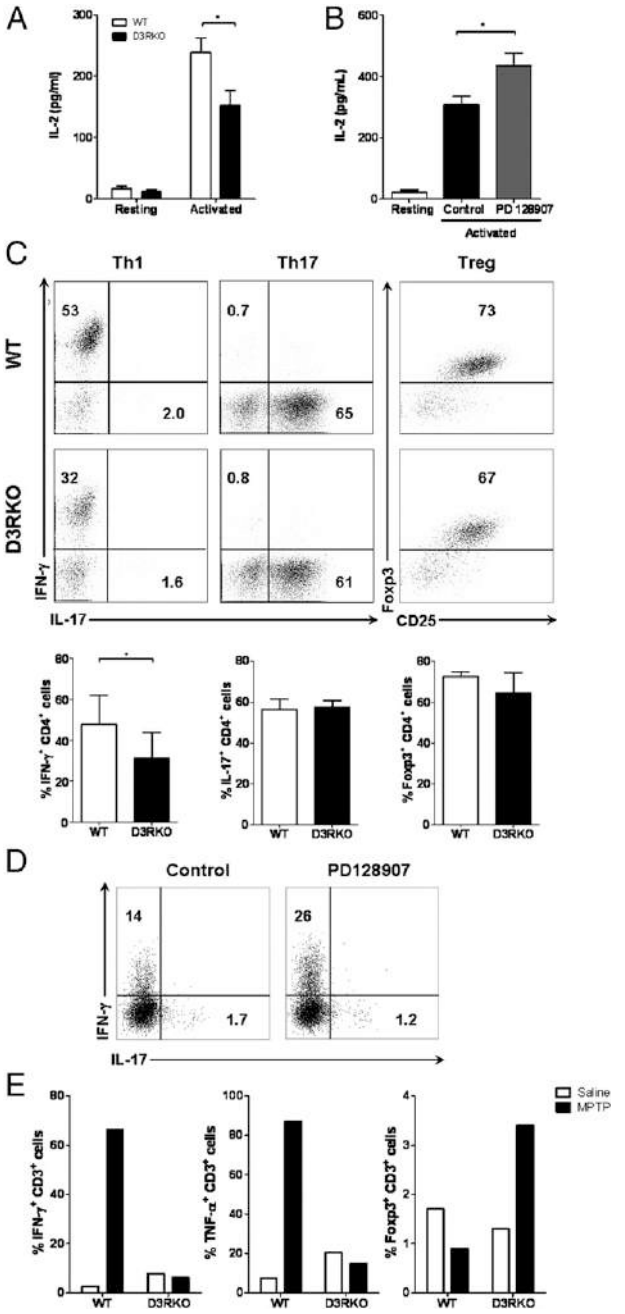

FIGURE 5. D3R expression on CD4+ T cell favors activation and polarization toward Th1 effector-phenotype. (**A**) WT or D3RKO CD4+ T cells were cultured in the absence (resting) or the presence (activated) of anti-CD3ε and anti-CD28 mAbs for 24 h, and IL-2 release was assessed by ELISA. (**B**) WT CD4+ T cells were cultured as in (A) either in the absence (control) or the presence of 50 nM PD128907, a specific D3R agonist. *$p < 0.05$ (A, B). (**C**) WT or D3RKO naive CD4+CD25− T cells were stimulated with spleen DCs in the presence of soluble anti-CD3ε and polarizing conditions toward Th1 (*left panels*), Th17 (*middle panels*), and Treg (*right panels*) phenotypes, as indicated in *Materials and Methods*. After 7 d, cells cultured on Th1 and Th17 conditions were restimulated, and IL-17 and IFN-γ production were assessed on the CD4+ population by flow cytometry. Treg cultures were evaluated for Foxp3 expression on the CD4+CD25+ population after 6 d of culture. Representative dot plots from one of three independent experiments are shown (*upper panels*). Numbers on plots indicate the percentage of CD4+ T cells inside corresponding quadrant. Graphs show the percentage of cytokine-producing or Foxp3-expressing CD4+ T cells on each condition (*bottom panels*). *$p < 0.05$ by paired *t* test. (**D**) WT naive CD4+CD25− T cells were stimulated with anti-CD3ε and anti-CD28 mAbs under Th1 conditions either in the absence (control) or the presence (PD128907) of 50 nM PD128907. After 4 d, cells were restimulated, and IL-17 and IFN-γ production was assessed on the CD4+ population by flow cytometry. Numbers on plots indicate the percentage of CD4+ T cells inside corresponding quadrant. (**E**) WT or D3RKO mice were i.p. injected with either PBS (saline) or MPTP. After 5 d, mononuclear cells obtained from SN-containing brain sections were restimulated and assessed for IFN-γ, TNF-α, and Foxp3 expression on the CD3+ population by flow cytometry. Graphs show the percentage of cytokine-producing or Foxp3-expressing T cells on each condition. Data representative from five mice per group are shown.

CD4+ T cell differentiation toward Th1 phenotype, an important inflammatory phenotype involved in PD physiopathology.

Studies performed by the group of Levite and coworkers (22, 29) using human T cells have suggested that D3R stimulation in resting T cells results in augmented production of TNF-α and activation of β_1 integrins such as VLA-4 and VLA-5. Confirming the "stimulatory" role of D3R-mediated stimulation in resting T cells, Ilani et al. (33) have shown that pharmacologic stimulation of D3R in human resting T cells favors expression of VLA-4 on the cell surface. Interestingly, several studies have shown that stimulation of DARs in activated T cells inhibits T cell function, including T cell activation, proliferation, and cytokine secretion (see Ref. 17 and references cited therein). However, most of those studies have shown a number of DA-mediated inhibitory effects using high concentrations (>1 μM) that affect multiple DARs. In contrast, a study addressing the role of selective stimulation of D3R in human T cells have shown that pharmacologic stimulation of D3R in activated CD4+ T cells favors expression of the cell surface activation marker CD25 as well as production of the proinflammatory cytokine IFN-γ (33). In agreement with this latter study, our findings in this study show both genetic and pharmacologic evidences indicating that D3R stimulation on CD4+ T cells favors T cell activation and production of the proinflammatory cytokines IFN-γ and TNF-α (Fig. 5).

Expression of DARs in T cells, including D3R, is very dynamic and depends on the activation state, specific T cell subset, pathologic conditions, and treatments (15, 20, 22, 26, 28, 29, 40–44). In this regard and interestingly, a decreased D3R expression in PBLs obtained from PD patients has been observed, which correlates with the degree of disease severity (34). This decreased expression of D3R in total blood lymphocytes from PD patients could be due to altered D3R expression in a lymphocyte population different of CD4+ T cells. Another possibility is that D3R expression on CD4+ T cells decrease during PD progression, which could represent a compensatory mechanism. Altered expression of D3R on CD4+ T cells could change the responsiveness of these cells to D3R-mediated effects in vivo. To test the possibility that D3R expression on CD4+ T cells change during MPTP-induced PD in mice, we performed some experiments addressing this issue. Our results show no difference in D3R expression in CD4+ T cells obtained from MPTP-treated mice versus PBS-treated mice. The same results were observed when T cells were obtained from mononuclear cells infiltrating the CNS, from cervical lymph nodes (the lymph nodes which drain cerebrospinal fluid bearing CNS Ags), and from spleen (data not shown). Thus, these results rule out an altered responsiveness of CD4+ T cells to D3R-mediated effects in mice undergoing MPTP-induced PD. Moreover, these results suggest that decreased D3R expression observed in lymphocytes from PD patients is due to altered D3R levels in another cell population different of CD4+ T cells. In this regard, it is necessary in the future to determine D3R expression, both at the mRNA level and at the protein level, in CD4+ T cells

and other cell subpopulations contained in the whole-blood lymphocyte population obtained from human PD patients. Moreover, to validate similarities and differences associated to the T cell response involved in PD between human PD and animal models of PD, it also would be useful in the future to determine D3R expression in $CD4^+$ T cells from mice undergoing PD in other models different from that induced by MPTP, such as those induced by other drugs (i.e., paraquat, rotenone, and 6-hydroxidopamine) or those induced by genetic approaches (i.e., α-synuclein and LRRK2) (45).

Interestingly, Joyce et al. (46) have shown that treatment with low doses of pramipexole, a drug characterized as a D3R agonist (47), has a neuroprotective effect in DAergic neurons of MPTP-treated mice. However, pramipexole resulted also in a downregulation of the DA transporter (DAT) (46), suggesting a relationship between D3R-induced signaling and DAT expression. Because MPTP-mediated intoxication involves specific uptake of 1-methyl-4-phenylpyridinium (MPP^+) by DAT, it is likely that pramipexole-induced neuroprotection is due to a reduced uptake of the toxic cation MPP^+ by DAergic terminals, making them less susceptible to MPTP-induced degeneration. Attenuated MPTP-induced neuronal loss in D3RKO mice observed in this study cannot be attributed to altered MPP^+ uptake by DAergic neurons or to decreased MPTP oxidation by glial cells, because transfer of WT $CD4^+$ T cells into D3RKO mice results in a MPTP-induced degeneration of DAergic neurons similar to WT mice (Fig. 4).

Several lines of evidence suggest a causal relationship between neuroinflammation and neuronal loss in PD (2, 38, 48, 49). In this context, microglial activation in the SN is a prominent pathological feature of PD (2). Accordingly, our results show consistent microglial activation in mice bearing WT $CD4^+$ T cells when treated with MPTP (Figs. 2–4), correlating with loss of DAergic neurons in the SN. Importantly, MPTP treatment of mice bearing D3R-deficient $CD4^+$ T cells resulted in an attenuated activation of microglia in the SN, which resemble a homeostatic phenotype (Figs. 2–4), correlating with neuronal survival. Thus, our results suggest that D3R expressed on $CD4^+$ T cells plays a role on microglial function, which has a consequent effect on DAergic neuron survival.

Our results show that D3R deficiency on $CD4^+$ T cells results in impaired capability to acquire the functional Th1 effector phenotype (Fig. 5). In agreement with these results, other authors have shown that pharmacologic stimulation of D3R in human $CD4^+$ T cells results in enhanced IFN-γ production (33). Importantly, IFN-γ has a critical role in stimulating and maintaining glial cell activation during PD, favoring chronic neuroinflammation (35). In contrast, WT and D3RKO $CD4^+$ T cells display similar capability to acquire phenotypes Th17 or Tregs (Fig. 5). Because D3R deficiency on $CD4^+$ T cells results in a strong neuroprotection in MPTP-induced PD and D3R expressed on $CD4^+$ T cells is involved in Th1 differentiation, but not in Tregs or Th17, our data suggest a key role for Th1 cells in PD. Moreover, these data suggest that changes in D3R expression on $CD4^+$ T cells can regulate the proinflammatory activity of $CD4^+$ T cells in PD.

Importantly, our results show that expression of D3R specifically in $CD4^+$ T cells results in destruction of ∼40% TH^+ cells in the SN when mice are treated with MPTP. This result was observed in two different systems: when WT $CD4^+$ T cells were transferred into two different mice strains refractory to MPTP-induced DAergic neurodegeneration, RAG1KO mice (Fig. 3B) and D3RKO mice (Fig. 4). Nonetheless, when the whole preparation of splenocytes obtained from WT mice were transferred into RAG1KO mice, these animals became extremely susceptible to MPTP-induced destruction of TH^+ cells in the SN (loss of ∼60% of TH^+ cells in the SN; see Fig. 3A). The fact that RAG1KO mice became quantitatively more susceptible to MPTP-induced destruction of TH^+ cells when transferred with WT splenocytes than when transferred with WT $CD4^+$ T cells (cotransferred with WT splenocytes depleted of $CD4^+$ T cells) suggests that a population different from $CD4^+$ T cells also contributes to MPTP-induced neurodegeneration, a notion supported by the lower ratio of non-$CD4^+$ T cells to $CD4^+$ T cells (3:1) used in the cell mixture compared with whole splenocytes (ratio 6:1). Another interesting and not yet explored implication of these results is that the expression of D3R in another population present in splenocytes, different of $CD4^+$ T cells may also contribute significantly to the MPTP-induced destruction of TH^+ cells. Considering that RAG1KO mice lack T and B cells but they have normal monocytes, macrophages, DCs, and other leukocyte populations present in splenocytes, D3R expressed in B cells and/or $CD8^+$ T cells could be responsible for the additional contribution to MPTP-induced neurodegeneration observed in RAG1KO mice transferred with WT splenocytes when compared with those RAG1KO mice transferred with WT $CD4^+$ T cells. In this regard, D3R expression has been described in $CD8^+$ T cells as well as in B cells (20, 26). Importantly, only $CD8^+$ T cells but not B cells have been found infiltrated into the SN of PD in human patients and mice treated with MPTP (6, 50). Thus, evidence suggests that D3R-expressing $CD8^+$ T cells present in splenocytes would potentiate the MPTP-mediated neurodegeneration mediated by $CD4^+$ T cells. Nevertheless, this hypothesis should be experimentally proved. However, because CD4- and TCR-β–deficient mice, but not CD8α-deficient mice, are resistant to MPTP-induced neurodegeneration (6), only $CD4^+$ T cells but not $CD8^+$ T cells seem to be strictly required to MPTP-induced PD.

Previous studies have shown an abrupt and strong decrease of stratial DA levels (>40-fold lower) after MPTP-induced PD in mice (6). In addition, D3R has the major affinity for DA (Ki ≈ 27 nM), followed by D5R (Ki ≈ 228 nM) and then D4R, D2R, and D1R (Ki ≈ 450, 1705, and 2340 nM, respectively) (51–54). Thus, despite the fact that $CD4^+$ T cells may express several DARs, it is likely that D3R would be selectively stimulated in $CD4^+$ T cells infiltrated into the CNS during MPTP-induced PD. According to this notion and taking in account the evidence shown in this article, we propose the following working model: initially, local intoxication of DAergic neurons with MPTP would generate an oxidative and inflammatory microenvironment in the SN. After an initial MPTP-induced loss of DAergic neurons and oxidation of α-synuclein and other Ags in the SN, an Ag-specific $CD4^+$ T cell response would be elicited against nitrated α-synuclein and another neoantigen, which would be presented in the cerebrospinal fluid-draining lymph nodes (cervical lymph nodes) (5, 7). Autoreactive naive $CD4^+$ T cells thus would be activated in the periphery where the presence of D3R would favor a potent activation and differentiation into Th1 inflammatory cells. Subsequently, these cells would leave lymph nodes and migrate into the CNS where they would be restimulated by microglial cells presenting neoantigens on the context of class II MHC (55). Furthermore, $CD4^+$ T cells infiltrated into the CNS would be exposed to low DA levels, which would selectively stimulate D3R, thus promoting high production of IFN-γ and TNF-α. These cytokines, in turn, would act synergistically over microglia favoring their activation and acquisition of the inflammatory phenotype M1 (35), specialized in secreting proinflammatory mediators, recruiting peripheral myeloid inflammatory cells, and thus promoting further destruction of DAergic neurons in the SN. Because α-synuclein aggregation or Lewy bodies formation, which contain oxidized CNS Ags, have been found in both drug- and genetically induced PD (5,

45), this working model would be applicable for drug-induced PD as well as for "spontaneous" genetically induced PD.

In conclusion, our results show a fundamental role of D3R expressed on $CD4^+$ T cells in the destruction of DAergic neurons during PD in a mouse model. These findings contribute to the knowledge of PD physiopathology and suggest relevant molecular and cellular targets for the treatment of this disorder.

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Disclosures

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