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Inhibition of dopamine receptor D3 signaling in dendritic cells increases antigen cross-presentation to CD8$^+$ T-cells favoring anti-tumor immunity

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ABSTRACT

Dendritic cells (DCs) display the unique ability for cross-presenting antigens to CD8$^+$ T-cells, promoting their differentiation into cytotoxic T-lymphocytes (CTLs), which play a pivotal role in anti-tumor immunity. Emerging evidence points to dopamine receptor D3 (D3R) as a key regulator of immunity. Accordingly, we studied how D3R regulates DCs function in anti-tumor immunity. The results show that D3R-deficiency in DCs enhanced expansion of CTLs *in vivo* and induced stronger anti-tumor immunity. Co-culture experiments indicated that D3R-inhibition in DCs potentiated antigen cross-presentation and CTLs activation. Our findings suggest that D3R in DCs constitutes a new therapeutic target to strengthen anti-tumor immunity.

1. Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells specialized in the initiation of adaptive immune responses by inducing the activation and supporting the differentiation of naive T lymphocytes toward effector T-cells (Banchereau and Steinman, 1998; Lanzavecchia and Sallusto, 2001). In this regard, among the different kinds of antigen-presenting cells, DCs display the unique ability to capture extracellular antigens, degrade them into small peptides and subsequently present these peptides on class I MHC molecules on the cell surface (Spel et al., 2013). This capability to carry out antigen cross-presentation makes DCs a central player in the induction of CD8$^+$ T-cells mediated responses.

Anti-tumor immune response involves the capture and processing of tumor-derived antigens by DCs, which subsequently migrate to draining lymph nodes and present peptides derived from tumor antigens on class I and class II MHC molecules to CD8$^+$ and CD4$^+$ T-cells, respectively (Bennett et al., 1998; Ridge et al., 1998). Upon specific recognition of tumor-derived antigens by TCRs, naive CD4$^+$ T-cells and naive CD8$^+$ T-cells become activated and differentiate toward T-helper-1 (Th1) and cytotoxic T-lymphocytes (CTLs) respectively. Whereas the main role of Th1 cells in the anti-tumor immune response is to support CTL differentiation and function (Ridge et al., 1998), CTLs may directly recognize and kill tumor cells expressing the specific tumor-derived antigen as peptide-MHC (pMHC) complexes (Bennett et al., 1998; Kawahara and Takaku, 2015). CTLs mediate the killing of tumor cells by secreting cytotoxic granules containing toxic proteins, such as perforin and granzyme (Schoenborn and Wilson, 2007). In addition, by secreting IFN-γ, CTLs may also potentiate the function of innate immune cells with relevant roles in anti-tumor immunity, including macrophages and natural killer (NK) cells (Pacheco et al., 2012). Thus CTLs play a central role in adaptive immune response against tumors.

Dopamine is typically recognized for controlling complex processes such as locomotion, cognition, hormone secretion, renal function and intestinal motility; however, recent evidence points dopamine as a key regulator of inflammation (Gonzalez et al., 2013; Pacheco et al., 2014; Prado et al., 2013; Shao et al., 2013; Torres-Rosas et al., 2014; Yan et al., 2015). Furthermore, a growing group of studies has shown a significant alteration of plasma dopamine levels in cancer patients (Pacheco et al., 2009), thus suggesting a relevant role of dopaminergic regulation in cancer. Dopamine exerts its effects by the stimulation of five different dopamine receptors (DARs), termed D1R-D5R (Sibley et

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al., 1993). All of these receptors are hepta-spanning membrane proteins that belong to the superfamily of G protein-coupled receptors. Based on their sequence homology, signal transduction machinery and pharmacological properties, DARs have been classified into two subgroups. D1R and D5R belong to type I DARs which often are coupled with stimulatory Gα subunits, while D2R, D3R, and D4R constitute type II DARs, which generally couple to inhibitory Gα subunits (Sibley et al., 1993). On the other hand, due to the fact that different DARs display different affinity for dopamine, differential stimulation of DARs is induced depending on dopamine levels. In this regard, D3R displays the highest affinity for dopamine (Ki ≈ 27 nM), followed by D5R (Ki ≈ 228 nM) and then D4R, D2R and D1R (Ki ≈ 450, 1705 and 2340 nM, respectively) (Pacheco et al., 2014).

Importantly, several studies have shown the expression of different DARs in immune cells, including DCs and T-cells (Pacheco et al., 2012; Pacheco et al., 2014; Pacheco et al., 2009; Pacheco et al., 2010). Pharmacological evidence obtained from studies carried out with human T-cells suggests that, among the five known DARs, both type I (D1R and D5R) and type II (D2R, D3R and D4R) receptors contribute to the regulation of T-cell functions. It has been suggested that stimulation of type I DARs expressed on human naive $CD4^+$ T-cells potentiates the production of Th2 cytokines (Nakano et al., 2009). Other investigators have shown that stimulation of type I DARs on human regulatory T-cells (Tregs) can decrease IL-10 and TGF-β production and their suppressive activity (Cosentino et al., 2007), and that D4R stimulation on human T-cells promotes quiescence (Sarkar et al., 2006). In addition, there is evidence that stimulation of D2R and D3R in normal human resting T-cells favors the production of IL-10 and TNF-α, respectively (Besser et al., 2005), and that stimulation of D3R in resting T-cells favors activation of β1-integrins and adhesion to fibronectin, two critical events required for cell migration (Levite et al., 2001). Moreover, pharmacological evidence in mouse has indicated a relevant role for D3R-stimulation in $CD8^+$ T-cells migration *in vivo* (Watanabe et al., 2006). Importantly, D3R-stimulation in human and mouse activated $CD4^+$ T-cells potentiates IFN-γ production, a key cytokine for Th1 function (Contreras et al., 2016; Ilani et al., 2004). Pharmacologic stimulation of D3R in human T-cells also potentiates the expression of surface activation markers (Ilani et al., 2004). In agreement with these studies, we have recently reported genetic evidence indicating a potent effect of D3R-signaling attenuating Th2 differentiation and favoring Th1 and Th17 responses *in vivo* in several settings of inflammation (Contreras et al., 2016). In addition, we have obtained genetic evidence indicating that D5R-signaling was important for $CD4^+$ T-cell activation (Franz et al., 2015).

Of note, for integration of the final outcome of dopamine in T-cell physiology it is important to consider not only the different effects mediated by DARs expressed in T-cells, but also the different affinities of DARs for dopamine. Thus, low levels of dopamine would selectively stimulate high affinity DAR, including D3R and D5R, favoring T-cell activation, migration, and the development of Th1 and Th17 responses (Contreras et al., 2016; Franz et al., 2015; Watanabe et al., 2006). On the other hand, high dopamine levels would stimulate also low-affinity DARs, including D1R and D2R, which seem to be dominant over high-affinity DARs and exert immunosuppressive effects in T-cells and myeloid cells, and thereby attenuate T-cell mediated immunity (Pacheco et al., 2014; Pacheco et al., 2009; Torres-Rosas et al., 2014; Yan et al., 2015).

Regarding dopaminergic regulation in DCs, D1R and D5R are expressed at high levels on the cell surface, whereas D3R and D2R are poorly represented in this cell population (Prado et al., 2012). Experiments using transference of wild-type (WT) or knockout DCs into WT recipient mice demonstrated that D5R-signaling on DCs strongly potentiates production of IL-23 and in a lower extent IL-12, consequently favoring Th17 and Th1 responses *in vivo* (Prado et al., 2013; Prado et al., 2012). In addition, pharmacological evidence has indicated that the selective stimulation of D2R/D3R or selective antagonism of D1R/D5R on DCs favors Th1 differentiation, but impairing Th17 responses (Nakano et al., 2008). Thus, depending on the concentration of dopamine, the specific DARs expressed and the type of immune cell bearing DARs, this neurotransmitter may exert diverse effects in the immune response.

Previous works showing the expression of DARs on DCs, a cell type that plays a fundamental role in the induction of the anti-tumor adaptive immune response, together with evidence suggesting a relevant role for dopaminergic regulation in the physiopathology of cancer, prompted us to study the relevance of dopaminergic regulation of DCs function in a mouse model of cancer. Here, we focus in the DAR displaying the highest affinity for dopamine, the D3R. Interestingly, our results show that D3R-deficiency confined to DCs results in a stronger CTLs-mediated response with a pronounced reduction in the extent of tumor growth. Furthermore, mechanistic analyses indicate that inhibition of D3R-signaling in DCs favors antigen cross-presentation to $CD8^+$ T-cells, thus potentiating their activation and the efficacy of tumor immunity.

2. Methods

2.1. Animals

Eight- to 12-weeks-old male mice were used for all experiments. Wild-type (WT) C57BL/6 mice where purchased from Jackson Laboratories (Bar Harbor, ME). D3R-knockout (D3RKO) mice were kindly donated by Dr. Marc Caron. D3RKO mice were generated in the 129SvJ strain (Joseph et al., 2002) and then backcrossed for more than ten generations in the C57BL/6 genetic background. OVA-specific OT-I transgenic mice in the C57BL/6 genetic background and expressing a specific TCR for $H\text{-}2K^b/OVA_{257–264}$ were obtained from Jackson Laboratories. OVA-specific OT-II transgenic mice in the C57BL/6 genetic background and expressing a specific TCR for $I\text{-}A^b/OVA_{323–339}$ were gently donated by Dr. María Rosa Bono (Ureta et al., 2007). Housing, breeding, and manipulation of mice were carried out according to institutional guidelines at the animal facility of the Fundación Ciencia & Vida.

2.2. Generation of bone marrow derived DCs

Bone marrow-derived DCs from WT and D3RKO mice were prepared as previously described (Prado et al., 2012). Briefly, bone marrow progenitors were differentiated to DCs 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) during 5 d. DCs differentiation was routinely assessed obtaining >90% $CD11c^+$ cells. Differentiated DCs were loaded with 1 mg/ml of OVA protein for 24 h and maturated with 400 ng/ml LPS (Sigma Chemical Co., St. Louis, MO) during last 4 h either in the presence or absence of the D3R-selective antagonist PG01037 (10 nM; from Tocris, Bristol, UK) and then used for further experiments.

2.3. DCs vaccination and tumor challenge

Mice were subcutaneously inoculated in the right flank with 10^6 OVA-expressing B16-F10 melanoma cells. In same experiments, $OVA_{257–264}$ specific $CD8^+$ T-cells (10^5 OT-I cells per mice) were intravenously transferred at the sixth day after inoculation with melanoma cells. One day later, mice were intravenously vaccinated with OVA-loaded DCs (2×10^6 cells per mice). Tumor growth was monitored by quantifying the perpendicular tumor diameters with calipers. Mice were sacrificed when moribund or when the mean tumor diameter was ≥15 mm, according to the institutional bioethics. In some experiments, mice were intravenously vaccinated with OVA-loaded DCs (10^6 cells per mice) and the activation of endogenous OVA-specific $CD8^+$ T-cells was later determined in the spleen as indicated in the associated figure legend.

2.4. Tumor infiltrating lymphocytes analysis

Tumors were chopped in small fragments and digested with collagenase (5 mg/ml) and DNAse (5 μg/ml) for 30 min at 37 °C in a CO_2 incubator. Cells were passed through a cell-strainer and lymphocytes were enriched on a Ficoll gradient. OVA-specific TILs were analyzed based on the expression of CD3, CD8, and the OT-I-associated TCR chains Vα2 and Vβ5 as described (Hogquist et al., 1994).

2.5. Immunostaining analysis

To determine the surface expression of complexes composed by class I MHC presenting the $OVA_{257–264}$ peptide, cells were incubated with a phycoeritrine(PE)-conjugated monoclonal antibody recognizing the H2-K^b/SIINFEKL complex (Clone 25-D1.16; Biolegend, San Diego, CA, USA) at 4 °C for 2 h. To analyze the expression of the other surface molecules, cells were immunostained with the following fluorochrome-conjugated monoclonal antibodies at 4 °C for 30 min: allophycocyanin-conjugated anti-CD11c (clone HL3), FITC-conjugated anti-CD80 (clone 16-10A1), PE-conjugated anti-CD86 (clone GL1), FITC-conjugated anti-H2-K^b (clone AF6-88.5), FITC-conjugated anti-CD40 (clone 3/23), PerCP- or allophycocyanin-conjugated anti-CD8 (clone 53-6.7), PE-conjugated anti-Vα2 (clone B20.1) and/or allophycocyanin-conjugated anti-Vβ5 (clone MR9-4), all of them from BD Pharmingen. For intracellular cytokine staining of $CD8^+$ T-cells isolated from tumors or from tumor-draining lymph nodes, cell suspensions were stimulated with $OVA_{257–264}$ peptide (1 μg/ml) for 8 h in the presence of GolgiPlug (BD Biosciences) during the last 6 h. For intracellular cytokine staining of endogenous splenic OT-I cells from DCs-immunized mice, splenocytes were stimulated for 4 h with phorbol-12-myristate-13-acetate (PMA, 50 ng/ml, Sigma-Aldrich, St. Louis, MO, USA) and ionomycin (1 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) in the presence of brefeldin A (5 μg/ml, Sigma-Aldrich, St. Louis, MO, USA). After staining of surface markers, cells were washed with PBS, and then fixed and permeabilized using Cytofix/Cytoperm™ Fixation/Permeabilization solution set (BD Biosciences) according to the manufacturer's instructions. Afterwards, cells were incubated for 30 min with allophycocyanin-conjugated anti-IFN-γ (clone XMG1.2) from Biolegend (San Diego, CA, USA). All flow cytometry analyzes were performed by using a FACSCanto II flow cytometer and collected data processed using FlowJo version 6.4.7 (Tree Star, Inc.).

2.6. T-cell activation assays

WT or D3RKO DCs were loaded with OVA protein (1 mg/ml) or with OVA-derived peptides $OVA_{257–264}$ (0.1 ng/ml) or $OVA_{323–339}$ (200 ng/ml) (both from GenScript Corp., Piscataway, NJ), and then matured with LPS (400 ng/ml) in the presence or absence of PG01037 (10 nM), as indicated above. Afterwards, cells were washed, resuspended in fresh pre-warmed medium and co-cultured at indicated ratios (indicated in the figures) with purified transgenic OT-I $CD8^+$ T-cells or OT-II $CD4^+$ T-cells (10^5 T-cells/well). 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 co-culture supernatant after incubation for 24 h by ELISA (Prado et al., 2012).

2.7. Experimental Autoimmune Encephalomyelitis (EAE) induction and evaluation

WT C57BL/6 mice were injected s.c. with 50 μg of myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ 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 of *Pertussis* Toxin (Calbiochem, La Jolla, CA) on day 0 and 2 as described before (Prado et al., 2012). 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 D3RKO mice were pulsed with 5 μg/ml pMOG for 4 h and then transferred intravenously into WT C57BL/6 recipient mice 14 and 7 d before EAE induction as described before (Prado et al., 2012).

2.8. Statistical analysis

All values were expressed as mean ± SEM. Differences in means between two groups were analyzed by 2-tailed Student's *t*-test or, when data was not normally distributed, with a non-parametric Mann-Whitney *U* test. Comparison between multiple groups was analyzed using one-way ANOVA. When ANOVA showed significant differences, pairwise comparison between means was tested by Tukey's posthoc analysis. Differences between genotypes in survival or percentage of tumors reaching a determined volume were analyzed by the Long-rank (Mantel-Cox) test. P value ≤ 0.05 was considered significant. Analyses were performed with GraphPad Prism 6 software.

3. Results

3.1. D3R-deficient mice display a significant protection to the development of melanoma

We first attempted to determine the overall effect of D3R-signaling in the immune response to cancer. For this purpose, we compared the development of an aggressive mouse melanoma model, the B16-F10 tumor cell line expressing OVA (hereafter called B16-OVA), in D3R-deficient and WT mice. Although tumor growth was slightly delayed in the group of D3R-deficient mice when compared to WT mice (Fig. 1A), D3R deficiency resulted in a significantly prolonged lifespan after the inoculation of tumor cells (Fig. 1B). Because there was a strong dispersion in the kinetics of tumor growth among individuals of both experimental groups, the tumor growth was also evaluated as the percentage of animals bearing tumors reaching specific sizes with respect to the time, including tumors with volume ≤300 mm^3, ≤ 350 mm^3 and ≤400 mm^3. For all the cut off analyzed the results showed that, indeed, the general deficiency of D3R results in a significant delay in tumor growth using an aggressive model of melanoma (Fig. 1C).

3.2. Lack of D3R in DCs potentiates anti-tumor response

Since not only DCs, but also other important players of anti-tumor immunity express D3R, including B cells, $CD4^+$ and $CD8^+$ T-cells, and Natural Killer (NK) cells (Contreras et al., 2016; Franz et al., 2015; McKenna et al., 2002; Watanabe et al., 2006), we next designed experimental settings aimed to analyze the precise role of D3R confined to DCs in tumor immunity. Accordingly, we next performed a set of experiments in an antigen-specific system involving the transference of WT or D3R-deficient DCs into WT recipient mice bearing tumors. For this purpose, recipient mice were inoculated with B16-OVA melanoma cells, and then transferred with OT-I $CD8^+$ T-cells. Afterward, to promote the anti-tumor immune response we adoptively transfer OVA-pulsed WT or D3RKO DCs into recipient mice (Fig. 2A). Of note, in preliminary determinations of tumor growth we setup the conditions to obtain a suboptimal anti-tumor response in mice receiving *ex vivo* manipulated WT DCs. Indeed, the results show a significant delay in tumor growth only when mice received D3RKO, but not WT DCs loaded with a tumor-antigen (Fig. 2B). In addition, the tumor growth was also evaluated as the percentage of animals bearing tumors reaching a volume ≤200 mm^3 and ≤300 mm^3. For both cut off analyzed only mice receiving D3R-deficient DCs displayed a significant reduction in tumor development in comparison to control mice that did not receive *ex*

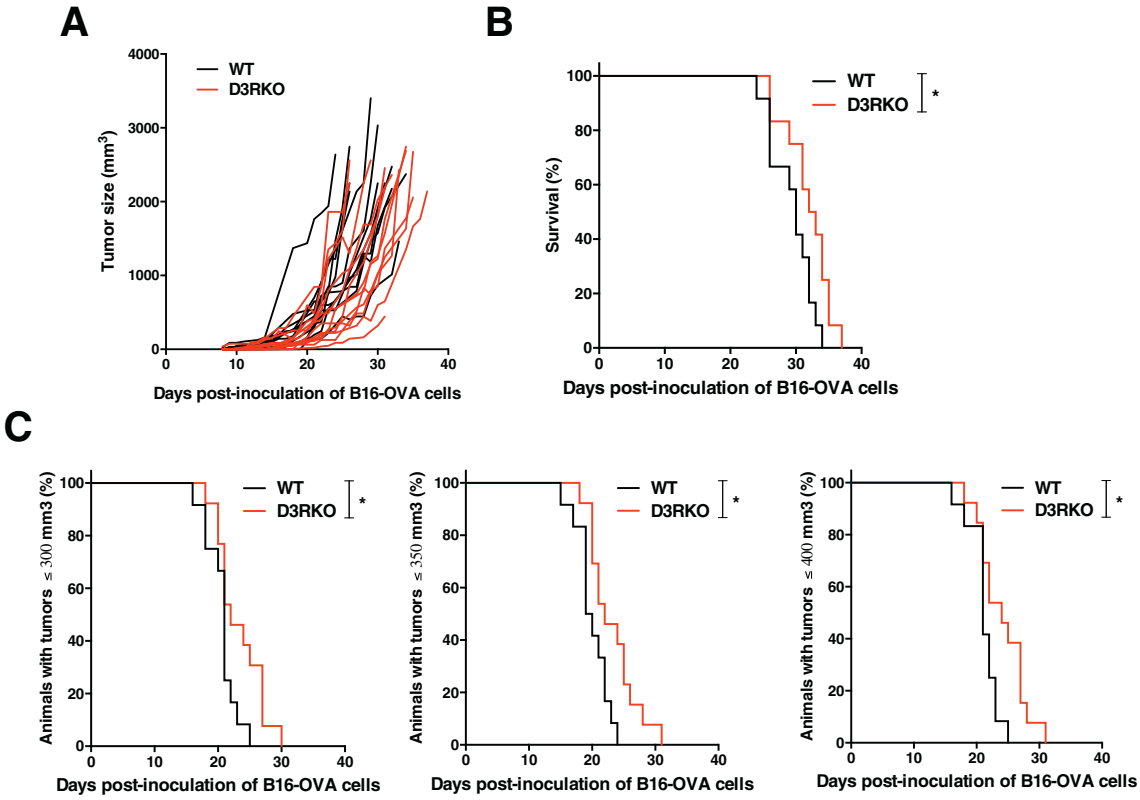

Fig. 1. D3R-deficiency results in delayed tumor growth in a mouse model of melanoma. 3×10^5 B16-OVA cells were intradermically injected into WT or D3RKO mice. Subsequently, tumor growth and survival were evaluated throughout the time-course of tumor development until tumors reached approximately 2000 mm^3. Data from 12 WT mice and 13 D3RKO mice are shown. (A) Individual tumor growth curves are shown. (B) The percentage of surviving mice was determined throughout the time-course of tumor development. (C) The percentage of mice bearing tumors with sizes ≤300 mm^3 (left panel), ≤350 mm^3 (middle panel) or ≤400 mm^3 (right panel) was determined throughout the time-course of tumor development. *, $p < 0.05$ by the Log-rank (Mantel-Cox) test.

vivo DCs treatment and to those mice transferred with WT DCs (Fig. S1). Taken together, these results indicate that the loss of D3R-signaling in DCs exacerbates tumor immunity.

To reinforce the conclusion obtained with genetic evidence (Figs. 2B and S1), we next performed similar experiments but using a pharmacologic approach to attenuate D3R-signaling in DCs. For this purpose *ex vivo* OVA-pulsed WT DCs were left untreated or treated with a highly-selective D3R antagonist, PG01037 (Grundt et al., 2005; Mason et al., 2010), and then i.v. transferred into tumor-bearing recipients. Similar to what was observed for mice receiving D3R-deficient DCs, tumor growth was significantly reduced in mice receiving WT DCs treated with a D3R-antagonist in comparison to those mice receiving untreated WT DCs or to those mice that did not receive *ex vivo* DCs (Fig. S2). Thus, these results represent pharmacological evidence confirming that the inhibition of D3R-signaling in DCs strengthens anti-tumor immune response.

3.3. D3R-deficiency in DCs favors $CD8^+$ T-cell response in vivo

Since CTLs are the main population of effector lymphocytes in the anti-tumor immune response, we next evaluated whether D3R-signaling in DCs may exert an effect in the CTL function. For this purpose we next analyzed the frequency of total or antigen-specific CTLs infiltrated into the tumor or in the tumor-draining lymph node of mice receiving DCs-therapy (see the scheme in Fig. 2A). The results show that, irrespective of the genotype of the therapeutic DCs transferred, the frequency of total tumor infiltrating lymphocytes (TILs) was increased when compared with the frequency of TILs in mice that did not receive DCs-therapy (Fig. 2C). However, when only those TILs specific to the tumor-antigen (OT-I cells) were considered in the analysis, we observed that mice receiving D3R-deficient DCs loaded with the tumor-antigen display a significantly stronger frequency of TILs than those mice receiving WT DCs (Fig. 2D). Interestingly, the frequencies of total CTLs or of those antigen-specific CTLs in the tumor draining lymph node were similar in all the experimental groups (Fig. S3). Thus, these results together indicate that inhibition of D3R-signaling in DCs loaded with a tumor-antigen strengthen the CTLs-response in the tumor.

Afterward, we wondered whether the inhibitory effect of D3R-signaling in DCs in the CTLs-mediated response was confined only to the context of tumor immunity or whether it can be reproduced in the $CD8^+$ T-cell mediated immune response against a foreign antigen. For this purpose, we analyzed the expansion of CTLs in tumor-free animals, which received WT or D3RKO DCs loaded with OVA and then, the activation of endogenous anti-OVA CTLs was quantified in the spleen (Fig. 3A). The results show that D3R-deficient DCs promote the development of a stronger CTL response than WT DCs, as we observed a higher frequency of antigen-specific CTLs producing IFN-γ *in vivo* (Fig. 3B and C). Together, these results indicate that the inhibition of D3R-signaling in DCs potentiates the antigen-specific CTL response not only in the context of tumor immunity, but in healthy mice as well.

3.4. D3R-signaling in DCs does not affect $CD4^+$ T-cell response

Since DCs play an important role in the induction of $CD4^+$ T-cell responses, we also determined the impact of D3R-signaling in DCs in the activation and function of $CD4^+$ T-cells. In this regard, we performed *in vitro* experiments in which WT or D3RKO DCs were loaded with the $OVA_{323–339}$ peptide and then co-cultured with antigen-specific $CD4^+$ T-cells purified from OT-II transgenic mice and subsequently the extent of T-cell activation was determined as IL-2 secretion in the culture supernatant. The results show that the extent of $CD4^+$ T-cell activation was similar when co-cultures were performed with WT or D3RKO DCs

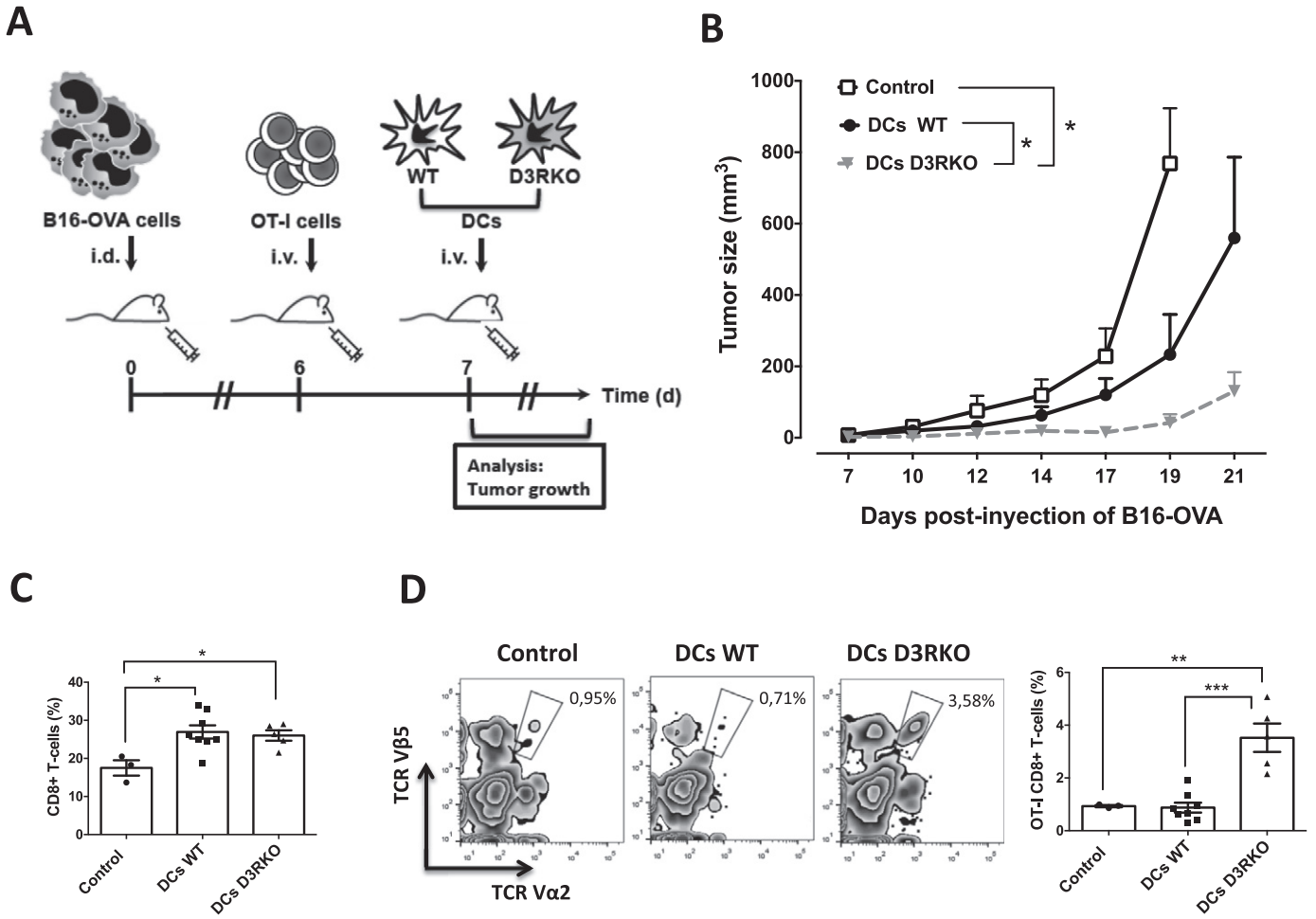

Fig. 2. Lack of D3R-signaling in DCs potentiates anti-tumor immunity. 10^6 B16-OVA cells were intradermally (i.d.) injected into WT recipient mice at day 0. On day 6 after tumor inoculation, 10^5 $OVA_{257–264}$ specific OT-I $CD8^+$ T-cells were i.v. administered. WT or D3RKO DCs were pulsed with OVA (1 mg/ml) overnight, and then matured with LPS (400 ng/ml) for 4 h, and subsequently i.v. injected (2×10^6/mouse) into the WT recipients 7 days after the inoculation with B16-OVA tumors. Afterwards, tumor growth was evaluated throughout the time-course of tumor development. (A) Scheme of experimental design. (B) Tumor size (n = 7–9 mice per group). Values represent mean ± SEM. *, $p < 0.05$ for comparison of the whole curves starting at day 10 by the Mann Whitney *U* test. (C–D) Tumor-infiltrating lymphocytes were extracted and the percentage of $CD8^+$ T-cells from total living cells (C) or the frequency of OT-I cells from living $CD8^+$ T-cells (D) were analyzed by flow cytometry. Values represent mean ± SEM. Representative density plots of OT-I $CD8^+$ T-cells analyzed are shown in left panels (D). Percentages of $TCRV\alpha2^+$ $TCRV\beta5^+$ cells from the living $CD8^+$ gate are indicated in each density plot. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by one-way ANOVA followed by Tukey's posthoc test (n = 3–8 mice per group).

(Fig. S4A), suggesting that D3R expressed in DCs does not play a relevant role in this process. Furthermore, to test the possibility that D3R expressed in DCs is relevant for $CD4^+$ T-cell response only when all the conditions *in vivo* are present, we next performed *in vivo* experiments using the Experimental Autoimmune Encephalomyelitis (EAE), a mouse model of $CD4^+$ T-cells-driven immune response (Goverman, 2009). When EAE was induced in WT and D3RKO mice, there were no significant differences in disease severity (Fig. S4B). Moreover, when DCs from WT or D3RKO mice were loaded *ex vivo* with the autoantigen involved (a peptide derived from the myelin oligodendrocyte glycoprotein, pMOG) and then i.v. injected in WT recipient mice in which EAE was induced, there was not differences in EAE severity (Fig. S4C). Thus, these results indicate that D3R-signaling in DCs does not regulate the $CD4^+$ T-cells response *in vivo*.

3.5. The inhibition of D3R in DCs favors the antigen cross-presentation

To gain insight in the underlying mechanism leading to stronger CTLs response exerted by the inhibition of D3R-signaling in DCs, we next performed analysis of $CD8^+$ T-cell activation *in vitro*. For this purpose, DCs were loaded with OVA, matured with LPS and treated or not with the highly selective antagonist for D3R, PG01037 (Grundt et al., 2005; Mason et al., 2010). Subsequently, these DCs were co-cultured with OT-I cells and the extent of T-cell activation was determined as the secretion of IL-2 in the supernatant, which was quantified by ELISA. The results show that the selective antagonism of D3R in DCs significantly exacerbated $CD8^+$ T-cells activation *in vitro* (Fig. 4A). Importantly, the potentiation of $CD8^+$ T-cell activation mediated by the treatment of DCs with PG01037 was in fact dependent on D3R inhibition, as this effect was abrogated when D3RKO DCs were used in this T-cell activation assay (Fig. 4B). Of note, when $OVA_{257–264}$ peptide was used to load DCs in T-cell activation assays, the extent of OT-I activation was not different between co-cultures using WT or D3RKO DCs (Fig. S5A) and was stronger than that observed when OVA protein was used as antigen (Fig. S5B). In this regard, it is important to consider that $OVA_{257–264}$ peptide does not require to be processed by intracellular proteases (as required by OVA protein) and it can directly form a complex with class I MHC molecule (Song et al., 1999). Since the effect observed in the potentiation of $CD8^+$ T-cell activation exerted by the inhibition of D3R-signaling in DCs was observed only when the antigen presented requires to be processed before presentation on class I MHC, we hypothesized that D3R-signaling was associated with antigen-cross presentation. To address this hypothesis we next performed experiments aimed to compare the extent of antigen cross-presentation

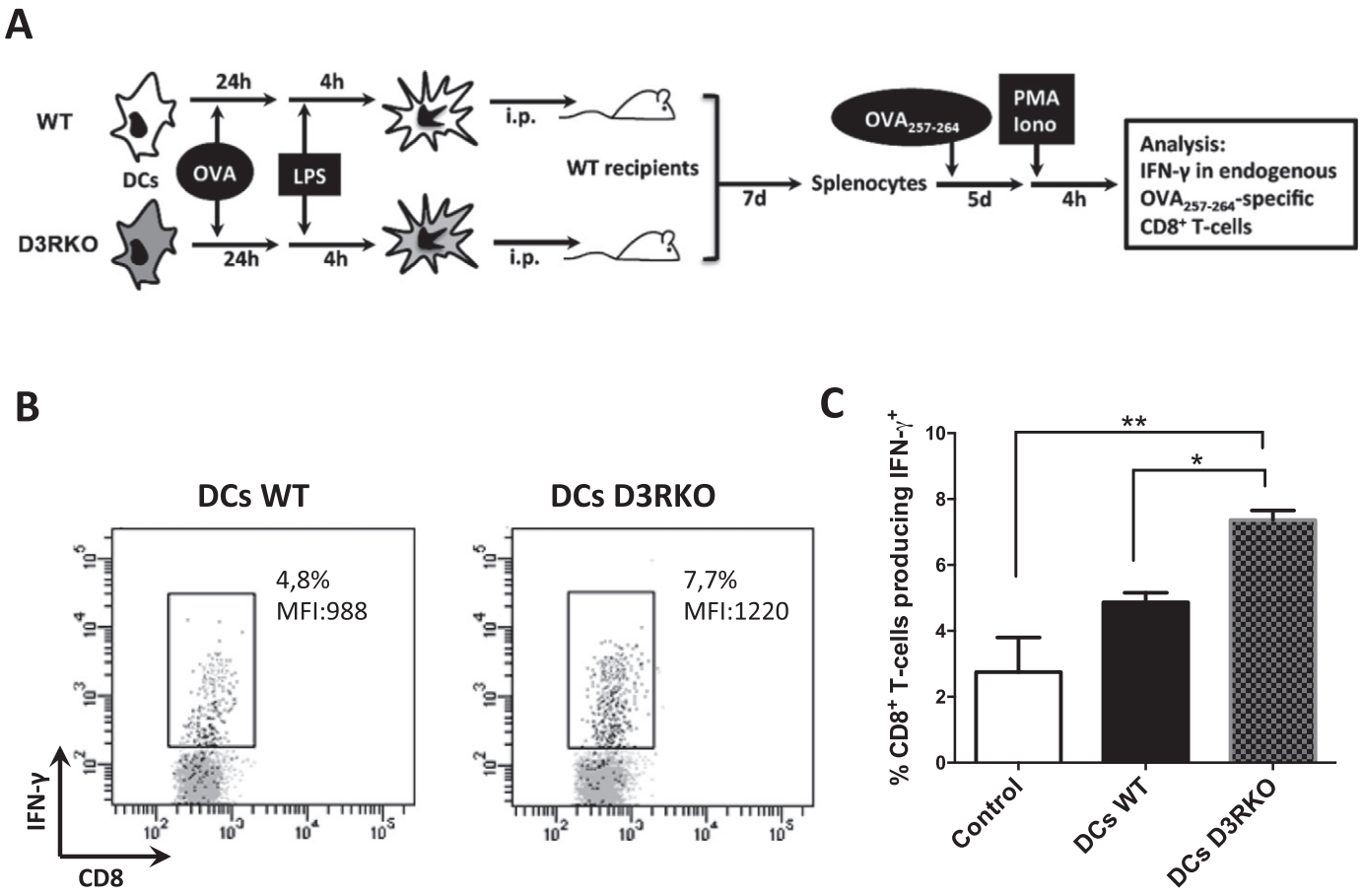

Fig. 3. D3R-deficiency in DCs favors a stronger $CD8^+$ T-cell response *in vivo*. WT or D3RKO DCs were pulsed with OVA (10 μg/ml) for 24 h, and then matured with LPS (400 ng/ml) for 4 h. Afterward, DCs were i.p. injected (10^6/mouse) in WT recipients. 7d later spleens were removed and splenocytes were pulsed with 10 nM $OVA_{257-264}$ peptide for 5d and restimulated with PMA and ionomycin for 4 h. Intracellular expression of IFN-γ in endogenous OVA-specific $CD8^+$ T-cells was determined by flow cytometry. (A) Scheme of experimental design. (B) Representative dotplots. The percentage and MFI associated to IFN-γ immunostaining in the $CD8^+$ population are indicated. (C) Results represented as frequencies of $CD8^+$ T-cells producing IFN-γ. Values represent mean ± SEM. Data from three independent experiments is shown. *$p < 0.05$; **$p < 0.01$ by one-way ANOVA followed by Tukey's posthoc test.

when D3R-signaling was abolished in DCs. For this purpose, WT or D3RKO DCs were treated or not with a D3R-selective antagonists and then pulsed with OVA protein. Afterward, the specific presentation of $OVA_{257-264}$ peptide in class I MHC was determined using a monoclonal antibody recognizing specifically the H2-K^b/$OVA_{257-264}$ complex (H2-K^b/SIINFEKL). The results show that, compared to WT DCs, D3RKO DCs basally display a higher degree of antigen cross-presentation and this process is not exacerbated after the treatment with a D3R-selective antagonist (Fig. 4C and D). Conversely, when WT DCs were treated with a D3R-selective antagonist, the extent of antigen cross-presentation was significantly increased (Fig. 4C and D). In contrast to OVA protein, $OVA_{257-264}$ peptide does not require prior processing to be presented on H2-K^b molecules (Song et al., 1999). Accordingly, we perform control experiments to determine how was the extent of H2-K^b/$OVA_{257-264}$ expression on DCs surface when antigen processing was not required. The results show that the extent of surface expression of H2-K^b/$OVA_{257-264}$ complexes on DCs surface was much higher when cells were incubated with $OVA_{257-264}$ peptide (Fig. S6) than when cells were incubated with OVA protein (Fig. 4C and D). These results indicate that differences in the surface expression of H2-K^b/$OVA_{257-264}$ complexes observed between WT and D3RKO DCs or between WT DCs left untreated and treated with PG01037 when OVA protein was administered (Fig. 4C and D) were due to differences in antigen processing. To rule out the possibility that the effect observed was due to a reduction of H2-K^b expression by D3R-signaling rather than an actual attenuation in the process of cross-presentation, we evaluated the levels of H2-K^b expression in WT and D3RKO DCs. Supporting the idea that D3R-signaling regulates antigen cross-presentation rather than the expression of H2-K^b, we observed no differences in the extent of H2-K^b expression of WT and D3RKO DCs (Fig. S7). Similarly, to discard the possibility of D3R-signaling was regulating co-stimulatory molecules, we evaluated the levels of expression of CD40, CD80 and CD86 in WT and D3RKO DCs and found no differences between genotypes (Fig. S7). Taken together these results indicate that inhibition of D3R-signaling in DCs favors the processes required for antigen cross-presentation and, thereby, potentiates the activation of $CD8^+$ T-cells.

4. Discussion

In this work, we present pharmacologic and genetic evidence demonstrating that the inhibition of D3R-signaling in DCs favors the antigen cross-presentation in class I MHC, thus promoting a stronger CTL response and consequently intensifying the tumor immunity. These findings not only constitute novel mechanistic knowledge in the fields of neuroimmunology and tumor immunology but also represent relevant data to consider in the design of therapies aimed to potentiate cellular immunity.

Our initial results showed that general D3R deficiency results in delayed tumor growth when using an aggressive model of melanoma (Fig. 1). These findings suggest that the general deficiency of D3R results in a more powerful immune response against tumor cells. In this regard, D3R expression has been described not only in DCs, but also in other immune cells with important roles in tumor immunity, including NK cells,

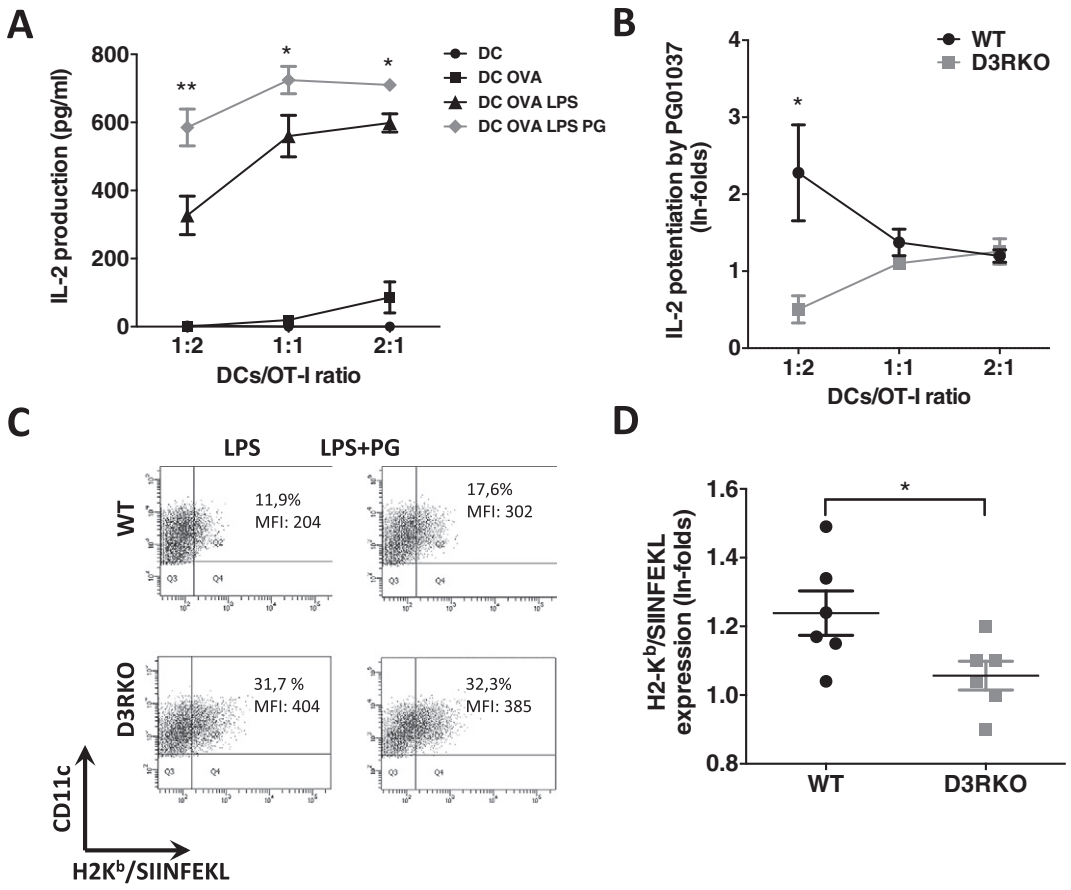

Fig. 4. Inhibition of D3R-signaling in DCs favors $CD8^+$ T-cells activation and cross-presentation. DCs from WT and D3RKO mice were pulsed with OVA (10 μg/ml) for 24 h, and then matured with LPS (400 ng/ml) for 4 h either in the presence or absence of a D3R-specific antagonist (PG01037, 10 nM). (A and B) Afterward, DCs were co-cultured with transgenic OVA-specific $CD8^+$ T-cells (OT-I) at different ratios. 24 h later, IL-2 was determined in the supernatant by ELISA. (A) IL-2 concentration in the co-cultures supernatant using WT DCs. (B) IL-2 production was determined in the presence or absence of PG01037 and the increase was represented as In-fold (IL-2 concentration reached in the presence of PG01037/IL-2 concentration reached in the absence of PG01037). (C and D) Surface expression of $H2K^b/OVA_{257–264}$ (H2-K^b/SIINFEKL) complexes was analyzed by flow cytometry. (C) Representative dotplots. The percentage and MFI associated to the surface expression of H2-K^b/SIINFEKL complexes are indicated. (D) Surface expression of H2-K^b/SIINFEKL complexes was determined in the presence or absence of PG01037 and the increase was represented as In-fold (Mean fluorescence intensity of H2-K^b/SIINFEKL in the presence of PG01037/Mean fluorescence intensity of H2-K^b/SIINFEKL in the absence of PG01037). (A, B and D) Data from six independent experiments are shown. Values represent mean ± SEM. To simplify the figure, significant differences in (A) are indicated only for the groups of DCs OVA LPS *versus* DCs OVA LPS + PG. *, $p < 0.05$; **, $p < 0.01$ by two-tailed unpaired Student's *t*-test.

$CD4^+$ and $CD8^+$ T-cells as well as macrophages (Contreras et al., 2016; Franz et al., 2015; McKenna et al., 2002; Watanabe et al., 2006).

A study addressing the role of D3R-signaling in $CD8^+$ T-cells has associated the stimulation of this receptor with improved $CD8^+$ T-cell responses. In this regard, D3R expressed in naive $CD8^+$ T-cells has been involved in migration, synergizing chemotaxis toward some key chemokines, including CXCL12, CCL19 and CCL21, and inducing the adhesion to fibronectin and ICAM1 through activation of integrins (Watanabe et al., 2006). On the other hand, D3R-signaling has been involved in the regulation of the acquisition of the functional phenotypes of $CD4^+$ T-cells: whereas it inhibits Th2 differentiation, Th1 and Th17 phenotypes are favored by D3R-signaling (Contreras et al., 2016; Franz et al., 2015). Since strong evidence has associated Th1 as well as $CD8^+$ T-cells responses in tumor immunity, it is expected that D3R deficiency in these lymphocyte subsets would result in impaired tumor immunity. Nevertheless, we observed here that the general D3R-deficiency results in stronger tumor immunity (Fig. 1), thus suggesting that D3R expressed in other cell populations is dominant turning down the immune response against tumors.

Interestingly, D3R has also been described in NK cells (McKenna et al., 2002; Zhao et al., 2013), macrophages (Gupta et al., 2011) and DCs (Nakano et al., 2008; Prado et al., 2012), three subsets of leukocytes with important roles in the immune response against tumors. A study addressing the role of D3R-signaling in the function of NK cells has shown pharmacologic evidence indicating that D3R-stimulation attenuates cytotoxic activity of NK cells (Zhao et al., 2013). On the other hand, the role of D3R-signaling in the function of DCs and macrophages has not been previously investigated. In this work, by using pharmacologic and genetic approaches we demonstrated that D3R-signaling confined to DCs attenuates the immune response against tumors (Fig. 2). Thus, at least partially, D3R-signaling confined to NK cells and DCs could explain the delayed development of melanoma observed in D3R-deficient mice in comparison to WT mice (Fig. 1).

Importantly, dopamine might regulate tumor development not only affecting anti-tumor immunity, but also angiogenesis (Sarkar et al., 2010; Sarkar et al., 2013). In this regard, by stimulating D2R in endothelial cells, dopamine inhibits the phosphorylation of VEGF receptor 2 and the consequent generation of new vessels in the tumor. Moreover, dopamine attenuates the mobilization of endothelial progenitors cells from bone marrow into the tumor site through D2R stimulation (Chakroborty et al., 2008). Thus, by the stimulation of D2R, dopamine might inhibit angiogenesis and, consequently, tumor development. With regard to the role of dopamine in the regulation of anti-tumor immunity, not only D3R but also type I DARs have been involved. Importantly, it has been shown that the stimulation of type I DARs inhibits the suppressive activity of both Tregs (Cosentino et al., 2007) and myeloid-derived suppressor cells (Wu et al., 2015). Despite the role of type I DARs on Tregs has not been studied in the context of tumors, it has been shown that dopamine (1 μM) or drugs selectively stimulating type I DARs in human Tregs inhibit the suppressive activity of these cells and

the production of the anti-inflammatory cytokines IL-10 and TGF-β (Cosentino et al., 2007). On the other hand, dopamine (5–50 μM) as well as type I DARs agonists attenuate IFN-γ-induced NO production by myeloid-derived suppressor cells obtained from tumor-bearing mice and from cancer patients and potentiate anti-tumor immunity in mouse models of cancer (Wu et al., 2015). Taken together these previous evidences and the present study, it is tempting to propose that, high dopamine levels attenuate tumor angiogenesis by stimulation of D2R and inhibit the suppressive activity of myeloid-derived suppressor cells and Tregs by stimulating type I DARs, thus dampening tumor development. Conversely, low dopamine levels promote a selective D3R-stimulation, which reduces antigen-cross presentation to $CD8^+$ T-cells, thereby favoring tumor development.

An intriguing question is what is the source of dopamine for DCs *in vivo*. In this regard, Mignini et al. have detected the presence of dopaminergic innervation in secondary lymphoid organs (Mignini et al., 2003), just the site where DCs present their antigens to T-cells. Another potential source of dopamine is the gut mucosa, a tissue that is highly populated by DCs and contains high levels of this neurotransmitter in steady-state (Magro et al., 2004; Magro et al., 2002). Of note, there is strong evidence suggesting that dopamine present in the gut comes from the intrinsic enteric nervous system (Pacheco et al., 2014). However, dopamine available for immune cells can also potentially come from non-nervous origin. For instance, Cosentino et al. have described that Tregs may synthesize and store dopamine, and thereby this subset of T-cells could represent a relevant source of dopamine *in vivo* (Cosentino et al., 2007). Moreover, we and other authors have found that mouse and human DCs may synthesize and store dopamine (Nakano et al., 2009; Prado et al., 2012). In this regard, it has been described that dopamine can be released upon DCs maturation (Prado et al., 2012) or during antigen-presentation (Nakano et al., 2009), exerting a potential autocrine stimulation of DARs. Thus, not only the innervation of secondary lymphoid organs and the intestinal mucosa may be important sources of dopamine for DCs *in vivo*, but also some leukocytes including Tregs and DCs.

It is important to note that in the context of cancer, the levels of dopamine are significantly increased in plasma (Lechin et al., 1990; Saha et al., 2001) and thereby it may become relevant as a source of dopamine for peripheral blood leukocytes, including DCs. However, our results show that lack of D3R-signaling in DCs was relevant favoring $CD8^+$ T-cells response not only in the context of cancer (Fig. 2), but also in tumor-free mice (Fig. 3). Therefore, it is likely that plasma is not a relevant source of dopamine for stimulation of D3R in DCs in our experimental conditions.

Our findings indicate that D3R-signaling attenuates the antigen cross-presentation in DCs (Fig. 4), thus reducing the stimulation of $CD8^+$ T-cells in response to an antigen captured in peripheral tissues. On the other hand, we have observed that D3R-signaling on DCs has no impact in $CD4^+$ T-cells response (Fig. S4). Accordingly, our findings suggest that D3R-signaling in DCs might be involve in favoring the ratio $CD4^+$ T-cells to $CD8^+$ T-cells. This regulation could reduce the efficacy of the immune response involved in the elimination of tumors (Cote et al., 2011; Hu et al., 2000) and certain pathogens such as *Toxoplasma gondii* (Grover et al., 2012; Guiton et al., 2009; Lutjen et al., 2006), which are eliminated more efficiently by CTL responses, while $CD4^+$ T-cells play a less relevant role. Thereby, the inhibition of D3R-signaling in DCs represents a promising therapeutic target to strengthen CTLs response required for elimination of some infectious agents and for anti-tumor immunity.

5. Conclusions

Our findings indicate that the genetic deficiency or the pharmacologic antagonism of D3R in DCs potentiate the extent of antigen cross-presentation on class I MHC molecules and the consequent activation of cytotoxic responses mediated by $CD8^+$ T-cells. Thus, the inhibition of D3R in DCs increases the efficiency of CTLs-mediated responses *in vivo* in the context of the immune response against an aggressive model of melanoma or even in the generation of immunity to a foreign antigen. This study demonstrates an important contribution of D3R-signaling in the regulation of DCs physiology and suggests a new therapeutic target to strengthen anti-tumor immunity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2016.12.014.

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