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Dopamine receptors D3 and D5 regulate CD4$^+$T-cell activation and differentiation by modulating ERK activation and cAMP production

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ABSTRACT

Dopamine receptors have been described in T-cells, however their signalling pathways coupled remain unknown. Since cAMP and ERKs play key roles regulating T-cell physiology, we aim to determine whether cAMP and ERK1/2-phosphorylation are modulated by dopamine receptor 3 (D3R) and D5R, and how this modulation affects CD4$^+$ T-cell activation and differentiation. Our pharmacologic and genetic evidence shows that D3R-stimulation reduced cAMP levels and ERK2-phosphorylation, consequently increasing CD4$^+$ T-cell activation and Th1-differentiation, respectively. Moreover, D5R expression reinforced TCR-triggered ERK1/2-phosphorylation and T-cell activation. In conclusion, these findings demonstrate how D3R and D5R modulate key signalling pathways affecting CD4$^+$ T-cell activation and Th1-differentiation.

1. Introduction

CD4$^+$ T-cells constitute central players in the adaptive immune response, as they regulate the function of several immune system cells and orchestrate the elimination of pathogens and neoplastic focus. CD4$^+$ T-cell activation is induced by antigen-presenting cells (APCs), presenting a cognate antigen attached on the major histocompatibility complex (MHC) in the presence of costimulatory molecules B7 that bind CD28 on the T-cell surface (Pentcheva-Hoang et al., 2004; Zheng et al., 2004). Depending on the combination of cytokines and other molecules produced during T-cell activation, naive CD4$^+$ T-cells may differentiate toward a particular effector phenotype, including T helper 1 (Th1), Th2 and Th17 (McGeachy and Cua, 2008; Zhou et al., 2009). Acquisition of the effector phenotype allows CD4$^+$ T-cells to promote the most appropriate kind of immune response for the elimination of a particular pathogen or tumour. Naive CD4$^+$ T-cells may also differentiate into regulatory T-cells (Tregs), which induce tolerance to cognate antigens (DiPaolo et al., 2007). Thus, activation and differentiation of CD4$^+$ T-cells determine the kind of immunity and tolerance to cognate antigens, thereby these processes must be tightly regulated. Accordingly, deregulation in these processes may be involved in deficient response against infectious agents or tumours, or also in autoimmune responses.

Dendritic cells (DCs) are the most potent APCs, which by directing the activation and differentiation of naive CD4$^+$ T-cells, are specialised in initiating immune responses (Lanzavecchia and Sallusto, 2001). Importantly, it has recently been described that DCs express the machinery necessary to synthesize and store the neurotransmitter dopamine (DA) in vesicular compartments (Nakano et al., 2009; Prado et al., 2012). Since DCs release DA upon antigen-presentation (Nakano et al., 2009), and T-cells express DA receptors (DARs) (Pacheco et al., 2009), DA may regulate activation and differentiation of T-cells. DCs are not the only source of DA for T-cells (Pacheco et al., 2009). Tregs also synthesize and accumulate DA in intracellular vesicles, which can be released to the extracellular space (Cosentino et al., 2007), where this neurotransmitter can exert effects over cells expressing DARs. Another source of DA is the autonomic innervation of secondary lymphoid organs, which could be relevant for T-cells during antigen presentation (Mignini et al., 2003). Moreover, the gastrointestinal mucosa, which plays a critical role in the induction of tolerance to

Abbreviations: Ab, antibody; APC, antigen presenting cell; BCA, bicinchoninic acid; DA, dopamine; DARs, DA receptors; DCs, dendritic cells; D*n*R, DA receptor *n*; D*n*RKO, D*n*R knockout; EAE, experimental autoimmune encephalomyelitis; ERK, extracellular signal-regulated kinase; FBS, foetal bovine serum; Foxp3, forkhead box P3; HRP, horseradish peroxidase; JNKs, c-jun N-terminal kinases; MHC, major histocompatibility complex; mAb, monoclonal Ab; MAPK, mitogen-activated protein kinases; MEK1/2, MAPK kinase for ERK1/2; pAb, polyclonal Ab; pMHC, peptide-MHC; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulphate; TCR, T-cell receptor; Th*n*, T helper *n*; Tregs, regulatory T-cells; WT, wild-type.

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food antigens and to commensal microbiota, constitutes a major source of DA available for immune cells. Importantly, deregulation of DA-mediated regulation of immunity in the gut context seems to be critical for maintaining the tolerance to innocuous antigens, as gut DA levels are strongly reduced in Crohn's disease and ulcerative colitis (Pacheco et al., 2014). Furthermore, under some pathophysiological conditions, such as those occurring in Multiple Sclerosis (Prado et al., 2012) or Parkinson's disease (Brochard et al., 2009; Reynolds et al., 2010), $CD4^+$ T-cells infiltrate into the central nervous system (CNS) where they are exposed to DA and other neurotransmitters. In fact, striatal DA levels are significantly increased and decreased in Multiple Sclerosis (Balkowiec-Iskra et al., 2007) and Parkinson's disease (Brochard et al., 2009) respectively, two neurodegenerative diseases where $CD4^+$T-cells have been found to play a critical role (Gonzalez et al., 2014). Another source of DA for T-cells migrating through the blood vessels is plasma DA, which in normal individuals reaches levels of 10 pg mL^{-1} (Saha et al., 2001a, 2001b). However, upon some pathological conditions, such as septic shock, plasma DA is strongly increased (Torres-Rosas et al., 2014).

DA exerts its effects by stimulating DARs expressed on the cell surface. Five DARs have been identified to date: D1R, D2R, D3R, D4R, and D5R (Sibley et al., 1993; Strange, 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 (Gαs), whilst D2R, D3R, and D4R constitute type II DARs, which generally couple to inhibitory Gα subunits (Gαi) (Sibley et al., 1993). On the other hand, due to the fact that different DARs present different affinities for DA, differential stimulation of DARs is induced depending on DA levels. In this regard, D3R displays 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) (Sunahara et al., 1991; Malmberg et al., 1993; Strange, 2001; Wu et al., 2005).

Pharmacological evidence obtained from a group of studies performed with human $CD4^+$ T-cells has suggested that differential stimulation of DARs may regulate cytokine production and T-cell activation and differentiation. In this regard, Ilani et al., (Ilani et al., 2004) have suggested that D3R stimulation decreases IL-4 and IL-10 synthesis and potentiates IFN-γ production, the hallmark cytokine of Th1 cells. Moreover, the same study has shown that D3R stimulation in human T-cells would potentiate expression of surface activation markers (Ilani et al., 2004). Accordingly, in vitro stimulation of peripheral blood mononuclear cells with DA at concentrations in which D3R is preferentially stimulated, promoted increased frequency of $CD4^+$ $CD69^+$ and $CD4^+$ IFN-γ^+ cells (Torres et al., 2005). In contrast, Saha et al., have shown that DA, at concentrations that should selectively stimulate D3R, inhibits human T-cell proliferation (Saha et al., 2001a, 2001b). On the other hand, Nakano et al., have suggested that stimulation of type I DARs expressed on human naive $CD4^+$ T-cells would contribute to the production of Th2 and Th17 cytokines (Nakano et al., 2009, 2011). Moreover, Besser et al., have shown evidence suggesting that stimulation of D2R, D3R and type I DARs in human T-cells obtained from healthy donors would regulate production of IL-10 and TNF-α (Besser et al., 2005). Cosentino et al., have suggested that stimulation of type I DARs on Tregs would decrease production of IL-10 and TGF-β (Cosentino et al., 2007). Furthermore, there is a group of in vivo studies indicating that systemic administration of a type I DARs antagonist attenuates Th17 differentiation in mice (Nakano et al., 2008, 2011; Nakagome et al., 2011). Despite these pharmacological evidences that give some clues about the role that DARs play in $CD4^+$ T-cells activation and differentiation, there are no genetic evidences demonstrating the relevance of these receptors and signalling pathways involved in the dopaminergic regulation of these processes.

The stimulation of T-cell receptor (TCR) by the peptide-MHC (pMHC) complex and B7-CD28 mediated co-stimulation triggers the activation of signalling pathways necessary to induce T-cell activation and subsequent differentiation. The signalling pathways that contribute to efficient activation of T-cells after antigen recognition include the PKC/Ca^{2+} pathway and the mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinases (ERKs), c-jun N-terminal kinases (JNKs) and p38. Together these signalling molecules promote the activation of transcriptional factors NF-κB, NF-AT and AP-1 complexes. The concerted stimulation of all of these pathways leads to efficient T-cell activation with expansion of antigen-specific T-cell clones and differentiation into effector cells (Pacheco et al., 2009).

Regarding regulation of TCR-triggered signalling by cAMP in T-cells, PKA (a protein kinase activated by cAMP) as well as cAMP induce inhibition of ERK phosphorylation (Ramstad et al., 2000) and of JNK activation (Harada et al., 1999), activate C-terminal Src kinase (Vang et al., 2001) and block NF-κB activation (Jimenez et al., 2001; Hershfield, 2005). It has been well established that all of these intracellular biochemical events induce a marked impairment on T-cell activation with inhibition of T-cell proliferation and cytokine production (Aandahl et al., 2002). Furthermore, recent studies have shown that increased cAMP levels may contribute to the differentiation of naive $CD4^+$T-cells toward Th1 and Th17 phenotypes (Li et al., 2012; Yao et al., 2013). Despite type I and type II DARs that are often coupled to stimulation and inhibition of intracellular cAMP production respectively (Sibley et al., 1993), they have also been found coupled to the regulation of phospholipase C and ion channel activity (Undie et al., 1994; Salter, 2003; Beaulieu et al., 2007) which could also modulate ERK activation. Moreover, the regulation of ERK1/2-phosphorylation has been shown to contribute to the decision of Th1/Th2 fate, in which a strong ERK1/2-activation is associated with inhibition of IL-4 production and of the consequent Th2 differentiation, thus favouring acquisition of the Th1 phenotype (Jorritsma et al., 2003; Saraiva et al., 2009; Chang et al., 2012). Therefore, by stimulating DARs, DA could regulate T-cell activation and differentiation.

Due to the key role of $CD4^+$ T-cells in the adaptive immune response and to the lack of genetic evidence demonstrating signalling pathways coupled to DARs in $CD4^+$ T-cells and their functional relevance in these cells, in this study we aimed to determine signalling coupled to D3R and D5R and their involvement in cell activation and differentiation by using a genetic approach. Our results show that D3R is coupled to the modulation of cAMP production and ERK phosphorylation, favouring T-cell activation and differentiation toward the Th1 effector phenotype. On the other hand, our data show that D5R-triggered signalling contributes to induce ERK phosphorylation without effect in cAMP production, which impacts in $CD4^+$ T-cell activation but not in Th1 differentiation.

2. Materials and methods

2.1. Animals

Eight- to 12-weeks-old mice of the C57BL/6 background were used for all experiments. Wild-type (WT) C57BL/6 mice where purchased from Jackson Laboratories. D5R-knockout (D5RKO) and D3RKO mice were kindly donated by Dr. David Sibley (Hollon et al., 2002) and Dr. Marc Caron (Joseph et al., 2002), respectively. Housing, breeding, and manipulation of mice were carried out according to institutional guidelines at the animal facility of the Fundación Ciencia & Vida which is compatible with the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Council of Europe No. 123, Strasbourg 1985).

2.2. Immunostaining analysis

To determine expression of surface molecules, $CD4^+$ T-cells were purified (see Section 2.6) and then immunostained with the following fluorochrome-conjugated monoclonal antibodies (mAbs) for 30 min:

allophycocyanin-conjugated anti-CD4 (clone GK1.5), PE-conjugated anti-CD25 (clone 3C7), both from Biolegend (San Diego, CA, USA). For intracellular cytokine staining, $CD4^+$ T-cells were stimulated for 4 h with phorbol-12-myristate-13-acetate (PMA, 50 ng mL^{-1}, Sigma-Aldrich, St. Louis, MO, USA) and ionomycin (1 μg mL^{-1}, Sigma-Aldrich, St. Louis, MO, USA) in the presence of brefeldin A (5 μg mL^{-1}, Sigma-Aldrich, St. Louis, MO, USA). After staining of surface markers, cells were washed with PBS, fixed, permeabilized with permeabilizing buffer (0.5% Saponin, 3% BSA in PBS) and then incubated for 30 min with allophycocyanin-conjugated anti-IFN-γ (clone XMG1.2) and PECy7-conjugated anti-IL-17 mAbs (clone TC11-18H10.1), both from Biolegend (San Diego, CA, USA). All flow cytometry analyses were performed by using a FACSCanto II flow cytometer and collected data were analysed by using FACSDiva software (both from BD Biosciences, San Jose, CA, USA).

2.3. RT-PCR

$CD4^+$ T-cells were purified (see Section 2.6), lysed and total RNA was extracted with the EZNA total RNA kit (Omega Bio-Tek, Norcross, GA, USA), treated with DNase using TURBO DNA-free kit (Ambion Inc., Austin, TX, USA), and 1 μg RNA was retrotranscribed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For D3R and β-actin traditional RT-PCR was performed using 1 μL cDNA (equivalent to 100 ng), 10 μL Go-Taq Green Master Mix 2× reagent (Promega, Madison, WI, USA), primers and water for a final volume of 20 μL. Forward and reverse primers were used at 0.5 μM each. PCR was carried out for 35 cycles with 95 °C melting (30 s), 57 °C annealing (45 s) and 72 °C extension (45 s). Primer sequences were as follows: β-actin forward: 5′-CAG CTT CTT TGC AGC TCC TT-3′, β-actin reverse: 5′-CCT GGA TGG CTA CGT ACA TGG C-3′; D3R forward: 5′-AGG TTT CTG TCA GAT GCC-3′, and D3R reverse: 5′-ATT GCT GAG TTT TCG AAC C-3′. In the case of D5R and GAPDH a 20-μL real-time PCR reaction included 1 μL cDNA, 10 μL Brilliant III Ultra Fast QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA), primers and water as indicated by the manufacturer's instructions. PCR was carried out for 40 cycles with 95 °C melting (20 s), 60 °C annealing (20 s) and 72 °C extension (20 s). All reactions were performed on a Stratagene Mx3000P (Agilent Technologies, Santa Clara, CA, USA). Primer sequences were as follows: D5R forward: 5′-CCC TAA CAT AAC TCA TCT TCT CC-3′, D5R reverse: 5′-TAA CCC TGC AAG TTC ATC CA-3′; GADPH forward: 5′-TCC GTG TTC CTA CCC CCA ATG-3′, and GADPH reverse: 5′-GAG TGG GAG TTG CTG TTG AAG-3′. Amplimer sizes were verified by electrophoresis on a 1.5% agarose gel following ethidium bromide staining.

2.4. Determination of intracellular cyclic AMP

$CD4^+$ T-cells were purified (see Section 2.6). Cyclic AMP (cAMP) determination was performed as described before (Pacheco et al., 2004). Briefly, cells (1×10^6 cells mL^{-1}) were washed twice and resuspended with PBS buffer. Cells were incubated with 50 μM zardaverine (a phosphodiesterase 4 inhibitor, Tocris, Bristol, UK) for 10 min at 37 °C and treated without (control) or with the adenylyl cyclase stimulator forskolin at 10 μM (Tocris, Bristol, UK) in the absence or presence of either 5 nM PD128907 (D3R selective agonist) or 20 nM SKF38393 (D1R/D5R agonist) (both from Tocris, Bristol, UK) for 45 min at 37 °C. Cells were lysed and levels of cAMP were measured using a *cAMP-Screen cAMP Immunoassay System* (Applied Biosystems, Foster, CA, USA) according to the manufacturer's protocol, using the lysed equivalent to 5×10^5 cells $well^{-1}$. Concentrations of agonists and antagonists used in functional assays (cAMP, ERKs, differentiation and activation) were selected according to the reported K_i values of the binding to their respective receptors.

2.5. Immunoblotting

To evaluate ERK1/2 phosphorylation during early TCR-triggered signalling (involved in T-cell activation), $CD4^+$ T-cells were purified (see Section 2.6). $CD4^+$ T-cells (2×10^6 cells mL^{-1}) were washed twice, resuspended in pre-warmed RPMI medium and stimulated with 1 μg mL^{-1} anti-CD3ε Ab (Biolegend, San Diego, CA, USA) in the presence or absence of either 50 nM PD128907 (Tocris, Bristol, UK) or indicated concentrations of DA hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) for 5 min. Cells were lysed with lysis buffer (0.5% SDS, 12.5 mM Tris–HCl pH 6.8, 2.5% glycerol, 0.0025% bromophenol blue and protease and phosphatase inhibitor cocktail (both from Roche, Mannheim, Germany)). For phospho-ERK1/2 analysis in late TCR-triggered signalling involved in Th1 differentiation, $CD4^+CD25^-$ naive T-cells sorted from splenocytes and lymph nodes were incubated under Th1 conditions (see below) during different time points. When indicated, the inhibitor of MAPK kinase for ERK1/2 (MEK1/2) PD 98059 (Tocris, Bristol, UK) was added 24 h after cell differentiation began. At indicated end time points, cells were washed twice with PBS and lysed with lysis buffer (see above). In both cases (T-cell activation experiments and differentiation experiments), protein concentrations were estimated using the bicinchoninic acid (BCA) method (Thermo Scientific, Rockford, IL, USA). Cell lysates (50 μg $sample^{-1}$) were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (PVDF, Thermo Scientific, Rockford, IL, USA), and diphosphorylated-ERK1/2 were detected using a mouse phospho-specific ERK1/2 mAb (1:10,000; Sigma-Aldrich, St. Louis, MO, USA) followed by HRP-conjugated goat anti-mouse IgG Ab (1:5,000; Rockland, Gilbertsville, PA, USA). Immunodetection was carried out with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA). Membranes were stripped and reprobed with rabbit anti-ERK1/2-specific pAb (1:10,000; Sigma-Aldrich, St. Louis, MO, USA) followed by HRP-conjugated goat anti-rabbit Ab (1:5,000; Rockland, Gilbertsville, PA, USA) and detected as described above. Protein bands were quantified and normalised relative to the loading control band with ImageJ software (National Institute of Health, Bethesda, MD, USA).

2.6. T-cell activation assays

Purification of WT, D3RKO or D5RKO $CD4^+$ T-cells from total splenocytes and lymph nodes was carried out by negative selection using magnetic beads-based $CD4^+$ T-cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were incubated in RPMI 1640 medium supplemented with 10% inactivated foetal bovine serum (FBS), 2.05 mM L-glutamine, 100 Units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids (all of them from Life Technologies, Grand Island, NY, USA) at 37 °C in a humid atmosphere of 5% CO_2. Cells were activated (2×10^5 cells $well^{-1}$) in plate-bound anti-CD3ε (50 ng $well^{-1}$) and anti-CD28 (50 ng $well^{-1}$) mAbs (Biolegend, San Diego, CA, USA) in the absence or in the presence of either the D3R selective agonist PD128907, the D1R/D5R antagonist SCH233390, the MEK1/2 inhibitor PD98059, the adenylyl cyclase stimulator forskolin or the phosphodiesterase 4 inhibitor Zardaverine (all of them from Tocris, Bristol, UK). When indicated, cells were also incubated in the presence of the $G\alpha_i$ protein inhibitor Pertussis Toxin 100 ng mL^{-1} (Calbiochem, San Diego, CA, USA). After 24 h in cell culture, T-cell activation was determined as IL-2 secretion in the culture supernatant by ELISA, as described before (Prado et al., 2012).

2.7. Induction of Th1 differentiation

Naive $CD4^+CD25^-$ T-cells were purified by cell-sorting using a FACSAria II (BD Biosciences, San Jose, CA, USA). Cells were incubated in IMDM medium supplemented with 10% inactivated foetal bovine

serum (FBS), 2.05 mM L-glutamine, 100 Units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (all of them from Life Technologies, Grand Island, NY, USA) at 37 °C in a humid atmosphere of 5% CO_2. Cells were stimulated (2×10^5 cells $well^{-1}$) with plate-bound anti-CD3ε (100 ng $well^{-1}$) and soluble anti-CD28 (2 μg mL^{-1}) for 4 days. IL-2 (10 ng mL^{-1}), IL-12 (10 ng mL^{-1}) and anti-IL-4 mAb (5 μg mL^{-1}), all of them from Biolegend (San Diego, CA, USA), were added to the cell culture to promote differentiation toward the Th1 phenotype, as described before (Reboldi et al., 2009). When indicated, the MEK1/2 inhibitor PD 98059 (Tocris Biosciences, Ellisville, MO, USA) was added. After 4 days, cells were restimulated with ionomycin and PMA in the presence of brefeldin A, and intracellular cytokine staining was performed as indicated above (see Section 2.2). To quantify the extent of Th1 differentiation, the percentage of IFN-γ^+ cells was determined in the $CD4^+$ T-cell population by flow cytometry.

2.8. Statistical analysis

All values were expressed as mean ± SEM. Differences in means between two groups were analysed by 2-tailed unpaired Student's *t*-test. Differences with p values < 0.05 were considered significant. (*) indicates significant differences between plotted values. (#) indicates when ratios of values obtained with different genotypes (knockout/WT) are significantly different from 1. All of the statistical analyses were performed using the GraphPad Prism 6 software.

3. Results

3.1. D3R-stimulation, but not D5R-stimulation, can regulate forskolin-induced increase of intracellular cAMP levels in $CD4^+$ T-cells

Despite D3R and D5R have been found previously in $CD4^+$T-cells by other authors (reviewed in (Pacheco et al., 2009)), first we aimed to confirm the presence of these receptors in purified mouse $CD4^+$T-cells. For this purpose, we analysed the presence of the transcripts for D3R and D5R in freshly purified $CD4^+$T-cells by RT-PCR. The results show that mouse $CD4^+$T-cells express the mRNAs codifying for the D3R and for the D5R (Fig. 1A). Although DARs are often coupled to modulation of intracellular cAMP production (Sibley et al., 1993), they also have been found coupled to other signalling pathways in some cell types (Undie et al., 1994; Salter, 2003; Beaulieu et al., 2007). Since the coupling of D3R and D5R to the modulation of adenylyl cyclase has not been yet analysed in murine $CD4^+$ T-cells, we next aimed to evaluate whether the stimulation of these receptors is coupled with the regulation of cAMP in these cells. Accordingly, WT, D3R-deficient or D5R-deficient $CD4^+$ T-cells were treated with the adenylyl cyclase activator forskolin in the presence or absence of the D3R-selective agonist PD 128907 or the D1R/D5R agonist SKF 38393 and intracellular cAMP levels were then determined. Our results show that the stimulation of D3R significantly decreased intracellular cAMP levels induced by forskolin in $CD4^+$ T-cells, an effect lost in D3R-deficient $CD4^+$ T-cells (Fig. 1B). It is important to note that when absolute values of cAMP levels obtained from non-stimulated WT or D3RKO $CD4^+$T-cells were compared, they were not significantly different and were in the range of 0.1 nM to 1 nM. Similarly, absolute values of cAMP levels observed in forskolin-stimulated WT or D3RKO $CD4^+$T-cells were similar and they were between 5 nM and 10 nM. In contrast to D3R stimulation, pharmacological D1R/D5R stimulation had no effects on intracellular cAMP levels neither in WT nor in D5R-deficient $CD4^+$ T-cells (Fig. 1C). We also assessed whether PD 128907 or SKF 38393 was able to induce increased intracellular cAMP levels in WT, D3RKO or D5RKO $CD4^+$T-cells in the absence of forskolin. Results show that there were not significant differences between untreated controls and treated with PD 128907 or SKF 38393 (Fig. S1). Thus, these results constitute pharmacologic and genetic evidence indicating that D3R is coupled to $G\alpha_i$ in mouse $CD4^+$ T-

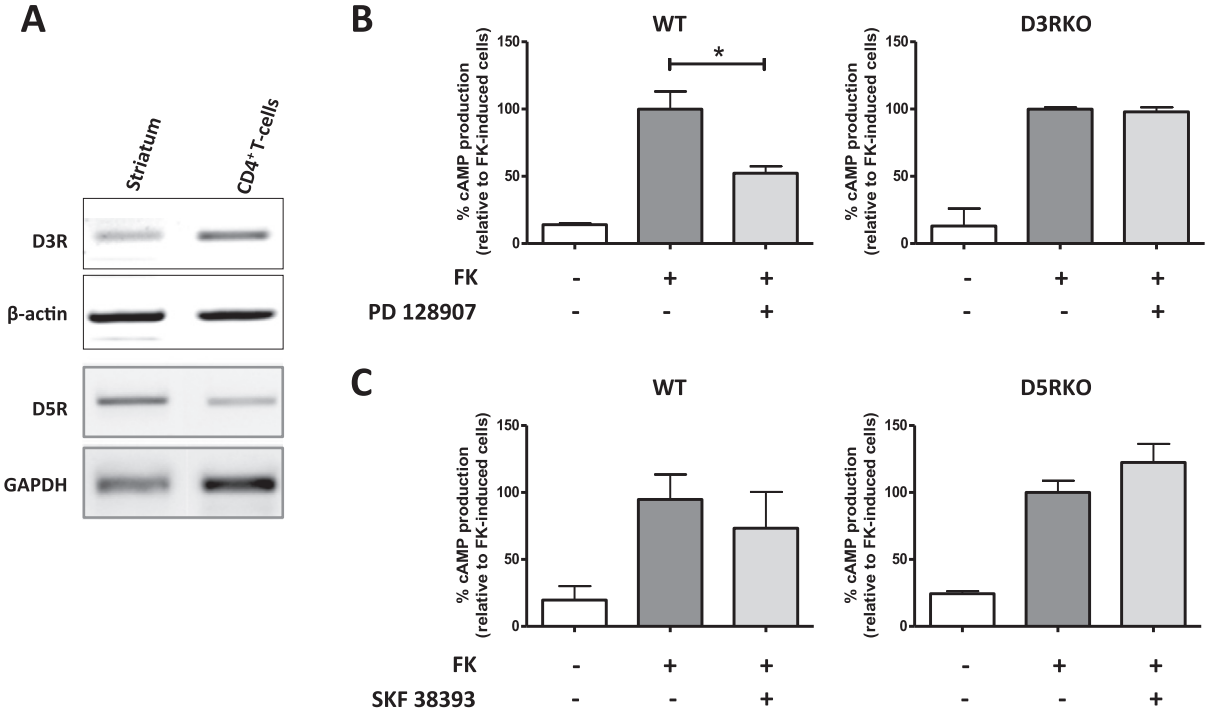


Fig. 1. $CD4^+$T-cells express both D3R and D5R, but only D3R stimulation reduces forskolin-induced cAMP levels in these cells. (A) $CD4^+$ T-cells were purified from WT mice and the expression of transcripts for D3R and D5R was evaluated by RT-PCR. Striatum was used as a positive control for expression of DARs. β-actin and GAPDH transcripts were analysed as controls. (B and C) $CD4^+$ T-cells obtained from WT, D3RKO and D5RKO mice were untreated (white bars) or treated with 10 μM forskolin (FK) in the presence (light grey bars) or absence (dark grey bars) of DAR agonists for 45 min. Afterward, cells were lysed and intracellular cAMP levels were determined by an ELISA-based kit. (B) WT (left panel) or D3RKO $CD4^+$T-cells (right panel) treated with the D3R-selective agonist PD128907 at 5 nM. (C) WT (left panel) or D5RKO $CD4^+$ T-cells (right panel) treated with the D1R/D5R-agonist SKF38393 at 20 nM. Values are expressed as percentage of the control (FK). (B and C) Data corresponds to mean ± SEM from three independent experiments. *, $p < 0.05$.

cells, whereas D5R is not coupled to the modulation of adenylyl cyclase in these cells.

3.2. Stimulation of D3R and D5R expressed on CD4$^+$ T-cells modulates TCR-triggered ERK1/2 phosphorylation

TCR stimulation triggers several signalling pathways including the phosphorylation of the MAPKs ERK1/2, which is involved in T-cell activation and differentiation (Pacheco et al., 2009). According to the critical role of ERK1/2 activation in the physiology of CD4$^+$ T-cells, we next addressed the question of whether D3R or D5R stimulation modulates TCR-induced phosphorylation of ERK1/2. For this purpose we first analysed how increasing DA concentrations affect TCR-induced ERK1/2 phosphorylation during early signalling. Range of DA concentrations was chosen to stimulate D3R and D5R according to their Ki (≈27 nM and ≈228 nM) without affecting significantly to D4R, D2R and D1R (Ki ≈ 450, 1705 and 2340 nM, respectively) (Sunahara et al., 1991; Malmberg et al., 1993; Strange, 2001; Wu et al., 2005). Results show that 50 nM DA, a concentration affecting exclusively to D3R but not to D5R, decreased the TCR-induced ERK2 phosphorylation (Fig. 2A). However, TCR-induced ERK2 phosphorylation was not significantly altered when CD4$^+$ T-cells were treated with 500 nM DA, a concentration that stimulates both D3R and D5R. Similarly TCR-induced ERK2 phosphorylation was not affected by 5 nM DA, a concentration that probably is too small to stimulate D3R or D5R. Moreover, the treatment of CD4$^+$ T-cells with DA in the absence of TCR-triggered signalling did not modify significantly the ERK1/2 phosphorylation at any concentration used (Fig. 2A). In addition, the treatment of CD4$^+$ T-cells with DA did not affect significantly ERK1 phosphorylation at any concentration used, in the presence or absence of TCR-triggered signalling. According to this, only ERK2 phosphorylation was considered for analyses in next experiments addressing the modulation of TCR-induced ERK activation by DAR stimulation. Thus, these results suggest that D3R stimulation attenuates TCR-induced ERK2-phosphorylation, whilst D5R stimulation seems to favour ERK2 phosphorylation, counteracting the D3R-mediated inhibitory effect on this signalling pathway. To

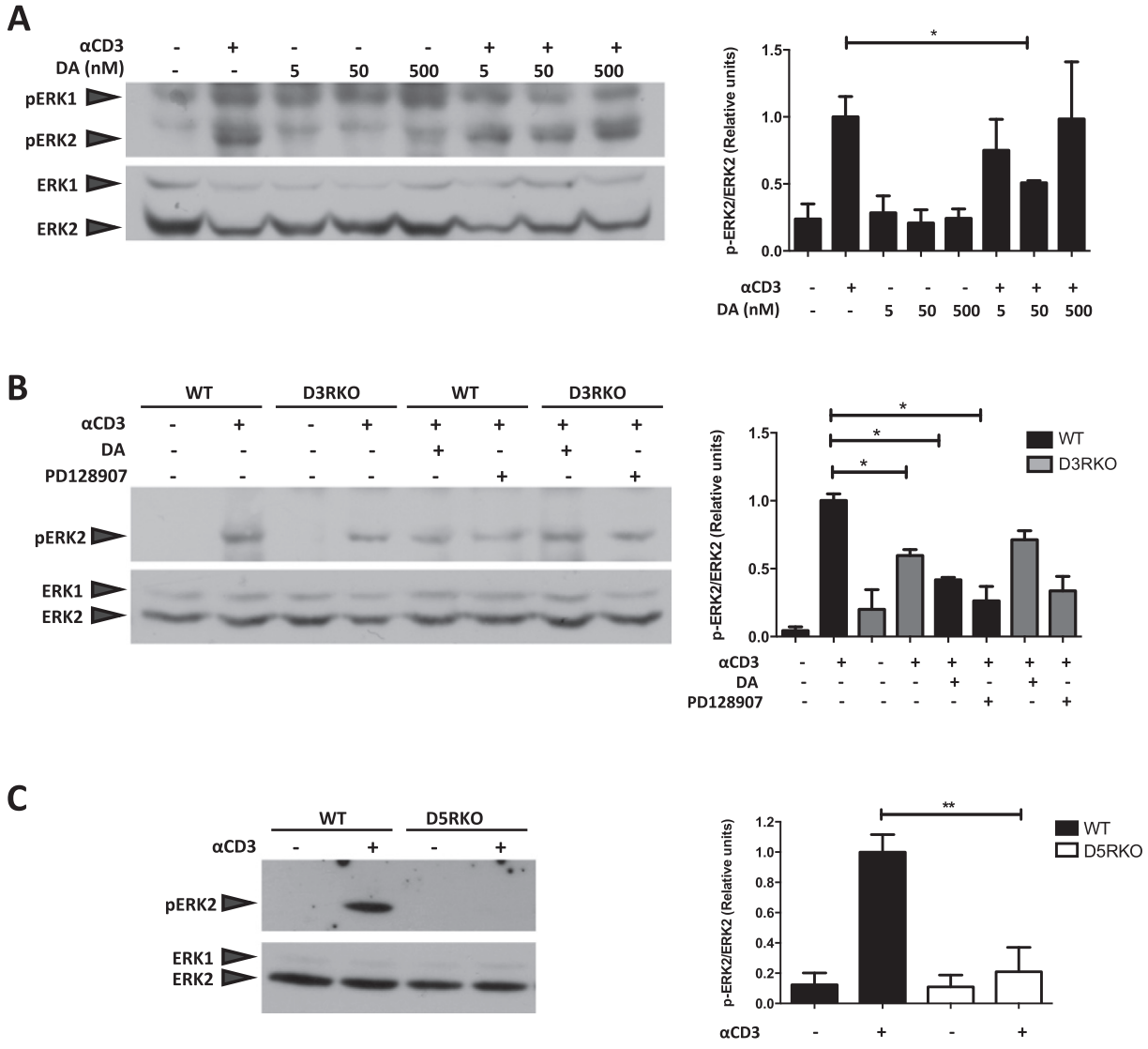


Fig. 2. Stimulation of D3R and D5R modulates phosphorylation of ERK2 in CD4$^+$ T-cells. CD4$^+$ T-cells were untreated or treated with 1 μg mL^{-1} anti-CD3ε mAb, in the absence or in the presence of DAR agonists for 5 min. Cells were lysed and the presence of diphosphorylated (pERK1/2) or total ERK1/2 (top and bottom panel, respectively) was analysed in protein extracts by *Western blot*. (A) CD4$^+$ T-cells obtained from WT mice were treated with DA at different concentrations in the presence or absence of anti-CD3ε mAb. (B) CD4$^+$ T-cells obtained from WT and D3RKO mice were treated with either 50 nM DA or 50 nM PD128907 in the presence or absence of anti-CD3ε mAb. (C) CD4$^+$ T-cells obtained from WT and D5RKO mice were untreated or stimulated with anti-CD3ε mAb. (A–C) Representative *Western blot* images are shown in left panels. Graphs on the right panels show densitometric quantification from four (A), two (B) and three (C) independent experiments. Values are the ratio of pERK2/ERK2 density of each treatment normalised to the ratio of pERK2/ERK2 density associated with the positive control (WT treated with anti-CD3ε mAb). Data represents mean ± SEM. *, $p < 0.05$; **, $p < 0.01$ (treatments versus positive control).

confirm this idea, we next performed analyses of TCR-induced ERK2 phosphorylation in WT or D3R-deficient CD4$^+$ T-cells stimulated by 50 nM DA or by the D3R-specific agonist PD 128907. Consistent with our previous results (Fig. 2A), stimulation with 50 nM DA or 50 nM PD 128907 attenuated the TCR-induced ERK2 phosphorylation in WT CD4$^+$ T-cells, but not in D3RKO CD4$^+$ T-cells (Fig. 2B). We also analysed the extent of ERK2 phosphorylation in WT or D3RKO CD4$^+$ T-cells stimulated with PD 128907 in the absence of TCR-stimulation, but not significant differences were observed between treatments (data not shown). Thus, these results indicate that D3R stimulation in CD4$^+$ T-cells attenuates TCR-induced ERK2 phosphorylation at early signalling. Similar to the analysis for D3R, we next performed experiments to confirm the involvement of D5R in the regulation of TCR-induced ERK phosphorylation using a genetic approach. Our data show that D5R-deficiency in CD4$^+$ T-cells results in a striking decrease of TCR-induced ERK2 phosphorylation (Fig. 2C). Due to the strong attenuation of TCR-induced ERK2 phosphorylation observed in D5R-deficient CD4$^+$T-cells, we increased the concentration of the anti-CD3ε mAb used to induce TCR stimulation. Results show that when the concentration of the anti-CD3ε mAb was enhanced up to 4-folds higher, the extent of ERK2 phosphorylation was similar in WT and D5RKO CD4$^+$ T-cells (data not shown), indicating that D5R affects the threshold of TCR-induced ERK2 phosphorylation. Taken together these results represent genetic and pharmacologic evidence indicating that D3R stimulation attenuates TCR-induced ERK2 activation during early signalling in CD4$^+$ T-cells, whereas D5R seems to be critical for the activation of this TCR-induced signalling pathway.

3.3. Stimulation of D3R and D5R contributes to the efficient activation of CD4$^+$ T-cells

Since D3R and D5R stimulation regulate cAMP production and ERK activation, two signalling pathways involved in the activation and differentiation of CD4$^+$ T-cells, we next addressed the question of whether these receptors modulate these critical processes. For this purpose we performed T-cell activation assays in which the extent of activation was determined as IL-2 secreted in the culture supernatant. Accordingly, we first determined how the genetic deficiency of D3R or D5R impact in T-cell activation. Our results show that both, D3R-deficiency or D5R-deficiency, result in impaired CD4$^+$ T-cell activation (Fig. 3A). Due to D5R was required for TCR-induced ERK2-phosphorylation (Fig. 2C), which is necessary for an efficient T-cell activation (Li et al., 1999), we next evaluated how the D1R/D5R antagonist SCH 23390 and the MEK1/2 inhibitor PD 98059 affect T-cell activation. According to the critical role of D5R in TCR-induced ERK2-phosphorylation, the D1R/D5R antagonist attenuated the T-cell activation (Fig. 3B). Furthermore, we confirmed that inhibition of the TCR–MEK1/2–ERK1/2-axis results in an impaired T-cell activation (Fig. 3C). Taken together these results and Fig. 2C indicate that, by favouring ERK2 phosphorylation, D5R contributes to induce an efficient CD4$^+$ T-cell activation. Furthermore, these results suggest that D3R-mediated attenuation of TCR-induced ERK2 phosphorylation (Fig. 2A and B) is not relevant for T-cell activation, as the genetic deficiency of D3R results in attenuated and not in exacerbated T-cell activation (Fig. 3A). According to this notion and to the fact that D3R stimulation

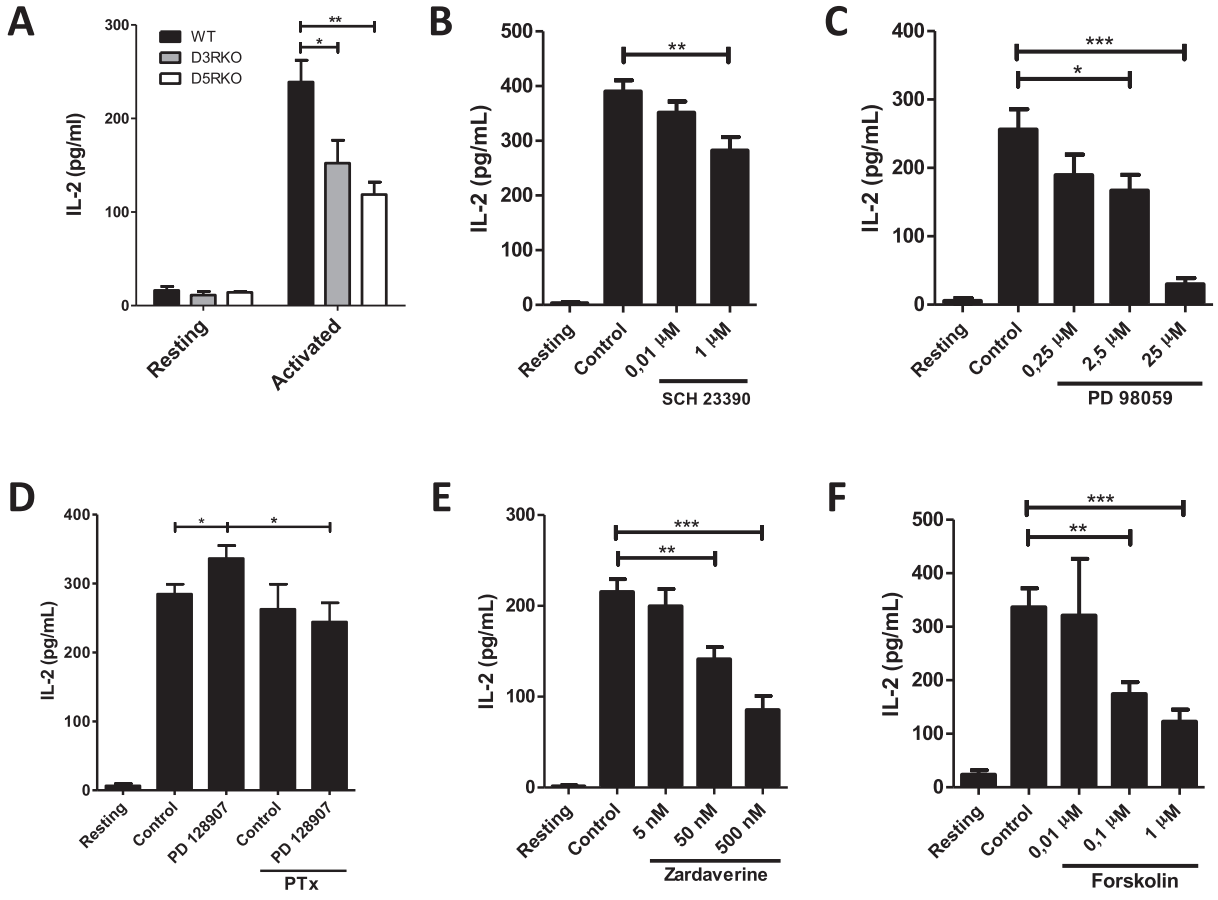


Fig. 3. Stimulation of D3R and D5R favours CD4$^+$ T-cell activation. CD4$^+$ T-cells obtained from WT (black bars), D3RKO (grey bars) or D5RKO (white bars) mice were untreated (resting) or activated with plate-bound anti-CD3ε plus anti-CD28 Abs (both at 50 ng well^{-1}) in the absence (control) or in the presence of different stimuli for 24 h and IL-2 secretion was determined in the culture supernatant by ELISA assay. (A) Comparison of CD4$^+$ T-cells from different genotypes. (B) WT CD4$^+$ T-cells were activated in the presence of the D1R/D5R antagonist SCH 23390 at 0.01 µM and 1 µM. (C) WT CD4$^+$ T-cells were activated in the presence of increasing concentrations of the MEK1/2 inhibitor PD 98059. (D) WT CD4$^+$ T-cells were activated in the presence of the selective D3R-agonist PD 128907 at 50 nM. When indicated, CD4$^+$ T-cells were pre-incubated with *Pertussis Toxin* (PTx) for 30 min previous to the induction of T-cell activation. (E) WT CD4$^+$ T-cells were activated in the presence of different concentrations of the PDE3/PDE4 inhibitor Zardaverine. (F) WT CD4$^+$ T-cells were activated in the presence of increasing concentrations of forskolin. Values represent mean ± SEM from at least three independent experiments. *, $p < 0.05$; **, $p < 0.005$ and ***, $p < 0.001$.

is also coupled to the inhibition of cAMP (Fig. 1B), we next evaluated how the D3R–Gα_i–cAMP-axis affects T-cell activation. In agreement with the inhibitory role of cAMP in T-cell activation (Aandahl et al., 2002), results show that the D3R-specific agonist PD 128907 potentiates the production of IL-2 (Fig. 3D). Furthermore, this effect was completely reversed when the Gα_i subunit was uncoupled using *Pertussis Toxin* (Fig. 3D). Additional experiments were performed to confirm that elevation of intracellular cAMP levels impacts negatively in T-cell activation. In this regard, we observed that both, the phosphodiesterase inhibitor zardaverine (Fig. 3E) and the adenylyl cyclase stimulator forskolin (Fig. 3F) inhibit T-cell activation in a dose-dependent manner. Taken together this data indicates that the D3R–Gα_i–cAMP-axis as well as the D5R–MEK1/2–ERK1/2-axis favour the activation of $CD4^+$ T-cells. However, the D3R–ERK2-axis is not relevant for T-cell activation.

3.4. D3R-mediated ERK2 phosphorylation contributes to the acquisition of Th1 phenotype by $CD4^+$ T-cells

Another important process in the physiology of $CD4^+$ T-cells is the differentiation into functional phenotypes. Importantly both, ERK activation and intracellular cAMP production have been involved in the regulation of $CD4^+$ T-cell differentiation (Jorritsma et al., 2003; Yao et al., 2013), thereby D3R and D5R could modulate this process. In this regard, we have recently described that D3R-deficiency results in impaired Th1 differentiation when it is induced in naive $CD4^+$ T-cells in the presence of WT splenic DCs (Gonzalez et al., 2013). Accordingly, we next analysed here how Th1 differentiation is modulated by D3R and D5R and their associated signalling pathways. To avoid interferences due to DCs, we used a protocol for induction of Th1 differentiation without DCs, as described before (Gonzalez et al., 2013). We first analysed how D3R or D5R deficiency affects Th1 differentiation. Results show that genetic D3R deficiency significantly impairs Th1 differentiation (Fig. 4A), whilst D5R deficiency was not relevant for this process (Fig. 4B). In agreement with these results, pharmacological evidence using the D3R-specific agonist PD 128907 in naive $CD4^+$T-cells has shown increased Th1 differentiation (Gonzalez et al., 2013). In addition, pharmacologic stimulation of naive $CD4^+$T-cells with the D1R/D5R agonists SKF 81297 (Fig. S2A) and SKF 38393 (data not shown) had no effect in Th1 differentiation. Moreover, when naive $CD4^+$T-cells were differentiated toward Th1 phenotype in the presence of 50 nM DA (a concentration that stimulates only D3R) but not with 500 nM DA (a concentration that stimulates both, D3R and D5R), the extent of Th1 differentiation was significantly increased (data not shown). With the purpose to explore the involvement of D3R–ERK2-axis and D3R–Gα_i–cAMP-axis in Th1 differentiation, we next performed experiments targeting ERK phosphorylation and cAMP levels. Accordingly, we evaluated the effect of the inhibition of MEK1/2 with PD 98059 at different time points during the differentiation of WT and D3RKO naive $CD4^+$ T-cells toward the Th1 phenotype. Interestingly, we observed that inhibition of ERK1/2 phosphorylation results in a significant increase in the extent of Th1 differentiation, with a maximal effect when ERK1/2 phosphorylation was inhibited after 24 h of the beginning (72 h of treatment with PD 98059) of the differentiation process (Fig. 4C). Furthermore, inhibition of ERK1/2-phosphorylation also increased significantly the extent of Th1 differentiation in D3RKO $CD4^+$ T-cells (Fig. 4C). In fact, the impairment in Th1 differentiation due to D3R deficiency was abolished when ERK1/2-phosphorylation was inhibited 24 h or later after the beginning of the differentiation process (Fig. 4C). According to these results, we next aimed to determine how is the kinetic of ERK1/2 phosphorylation in WT and D3RKO $CD4^+$ T-cells during the Th1 differentiation process. Our results show a peak of ERK2 phosphorylation at 36 h of Th1 differentiation in WT $CD4^+$ T-cells. Importantly, the extent of ERK2 phosphorylation was exacerbated in D3R deficient $CD4^+$T-cells (Fig. 5A), suggesting that this peak of ERK2 phosphorylation is responsible of the impaired Th1 differentiation displayed by D3RKO $CD4^+$ T-cells. According to this idea, we next analysed the peak of ERK2 phosphorylation induced at 36 h of Th1 differentiation in WT or D3RKO $CD4^+$ T-cells treated with the MEK1/2 inhibitor PD 98059 during the last 12 h. We found that the treatment with PD 98059 significantly decreased ERK2-phosphorylation in both D3RKO and WT cells (Fig. 5B), which is associated with the increase of Th1 differentiation when cells are treated with PD 98059 at 24 h observed in Fig. 4C. Thus, these results indicate that Th1 differentiation involves a peak of ERK2 phosphorylation at 36 h, which constitutes an inhibitory signal for Th1 differentiation, and which is exacerbated when $CD4^+$ T-cells lack D3R. With the purpose to explore the participation of D3R–Gα_i–cAMP-axis in Th1 differentiation, we next analysed the extent of Th1 differentiation of naive $CD4^+$ T-cells treated with the phosphodiesterase inhibitor zardaverine or with the adenylyl cyclase activator forskolin. Results show that the treatment of naive $CD4^+$ T-cells with agents that increase cAMP levels results in an enhanced extent of Th1 differentiation (Fig. S3). These results indicate that cAMP elevation during the process of differentiation contributes to the acquisition of Th1 phenotype. On the other hand, these results indicate that D3R–Gα_i–cAMP-axis is not relevant for Th1 differentiation. Taken together all of our results about Th1 differentiation, they indicate that D3R–ERK2-axis, and not D3R–Gα_i–cAMP-axis, potentiates the acquisition of Th1 differentiation.

4. Discussion

In this work, we present pharmacologic and genetic evidence demonstrating the role of D3R and D5R in the differential modulation of critical signalling pathways and consequent regulation of important processes of $CD4^+$ T-cell physiology. In this regard, we found a dual role for D3R in the T-cell physiology impacting in T-cell activation and differentiation. By coupling to Gαi protein, D3R stimulation reduces intracellular cAMP levels favouring T-cell activation, whilst late D3R-triggered signalling decreases the extent of ERK2 phosphorylation, thus contributing to the differentiation of $CD4^+$ T-cells into the inflammatory Th1 phenotype. On the other hand, D5R stimulation has a marked impact in early TCR-mediated signalling, favouring ERK2 phosphorylation and consequently contributing to an efficient T-cell activation. Considering these conclusions, we have integrated them and schematized a proposed working model in Fig. 6. This is the first study showing the signalling pathways coupled to D3R and D5R in purified murine $CD4^+$ T-cells and demonstrating the consequences of their stimulation in the physiology of these cells.

We have recently addressed the relevance of D3R-mediated regulation of $CD4^+$ T-cells physiology in vivo in a mouse model of Parkinson's disease (Gonzalez et al., 2013). Importantly, several authors have shown a pivotal role of $CD4^+$ T-cells in animal models of Parkinson's disease, as lack of $CD4^+$ T-cells results in attenuated neurodegeneration (Benner et al., 2008; Bueno et al., 2008; Brochard et al., 2009). Moreover, the relevance of the participation of inflammatory Th1 cells in the physiopathology of Parkinson's disease has been proved in mouse (Reynolds et al., 2010; Gonzalez et al., 2013). In agreement with the present study, our previous work has shown that D3R-deficient $CD4^+$ T-cells release a lesser extent of IL-2 upon T-cell activation and they also display an attenuated capability to differentiate toward the inflammatory Th1 phenotype (Gonzalez et al., 2013). Furthermore, we have demonstrated that D3RKO mice as well as WT mice bearing D3R-deficient $CD4^+$ T-cells are resistant to neurodegeneration associated with Parkinson's disease (Gonzalez et al., 2013). Thus, our previous work has demonstrated a fundamental role of D3R expressed on $CD4^+$ T-cells favouring the neurodegenerative process in a mouse model (Gonzalez et al., 2013). On the other hand, regarding the relevance of D5R in vivo, we have previously addressed the role of this receptor in experimental autoimmune encephalomyelitis (EAE), a $CD4^+$ T-cells mediated autoimmune response against central nervous system antigens. In this regard, we have shown that D5R deficient mice display a strong attenuation in the disease severity when compared with WT mice (Prado et al.,

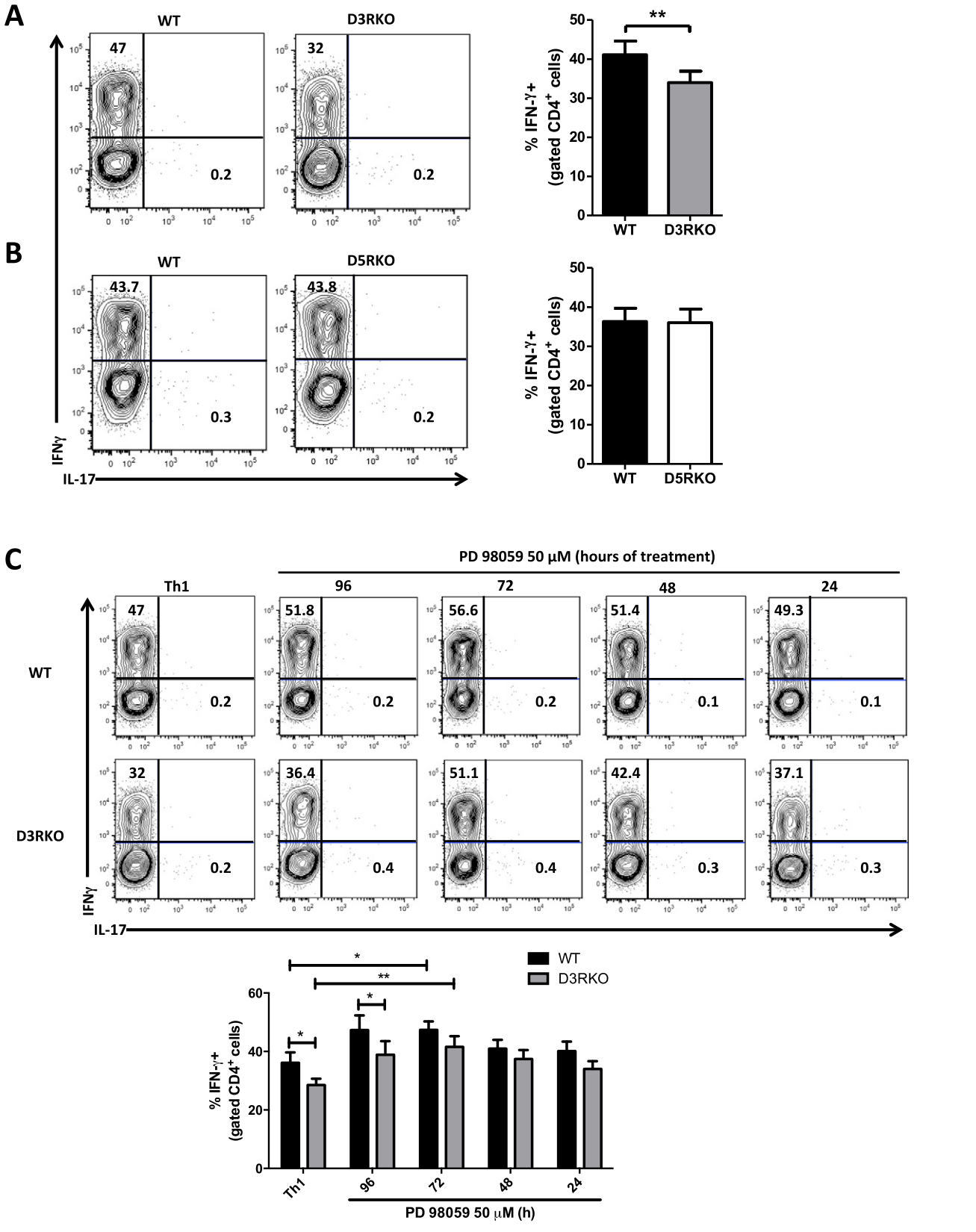
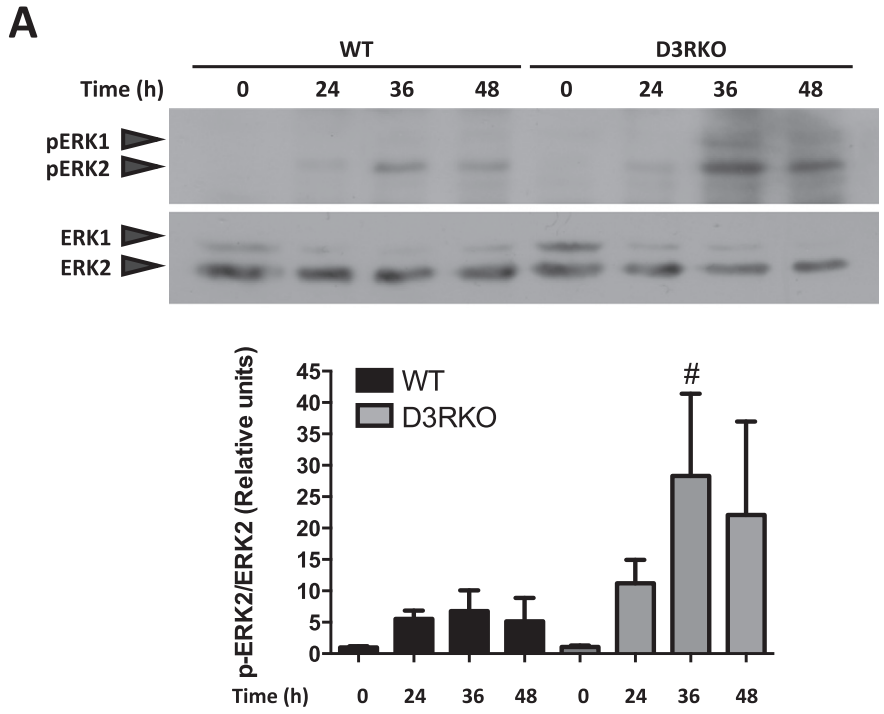


Fig. 4. D3R expression is required for efficient IFN-γ production by a mechanism dependent on MEK1/2. Sorted $CD4^+CD25^-$ naive T-cells obtained from WT, D3RKO or D5RKO mice were cultured under Th1 conditions for 4 days and cytokine secretion was evaluated by flow cytometry. WT and D3RKO (A) or D5RKO (B) naive T-cells were cultured in the presence of plate-bound anti-CD3ε mAb at 100 ng $well^{-1}$ and a cocktail of soluble Abs and cytokines (see Section 2) for 4 days. Then, cells were restimulated with PMA/Ionomycin in the presence of Brefeldin A for 4 h and intracellular cytokine analysis was carried out by flow cytometry. (C) WT and D3RKO naive T-cells were cultured under Th1 conditions for 4 days and 50 μM PD 98059 (MEK1/2 inhibitor) was added at different time points during the cell culture. The number of hours that differentiating T-cells remained with PD 98059 is indicated on the top. Cytokine production was measured by flow cytometry. (A–C) Representative dot plots for IL-17 versus IFN-γ production in the gated $CD4^+$ population are shown. Numbers inside quadrants indicate the percentage of IFN-γ^+ (top-left) and IL-17^+ (bottom-right) cells, respectively. Bar graphs represent mean ± SEM of percentage of IFN-γ^+ cells in the $CD4^+$ population. Data from eight (A) and nine (B and C) independent experiments is shown. *, $p < 0.05$; **, $p < 0.01$.



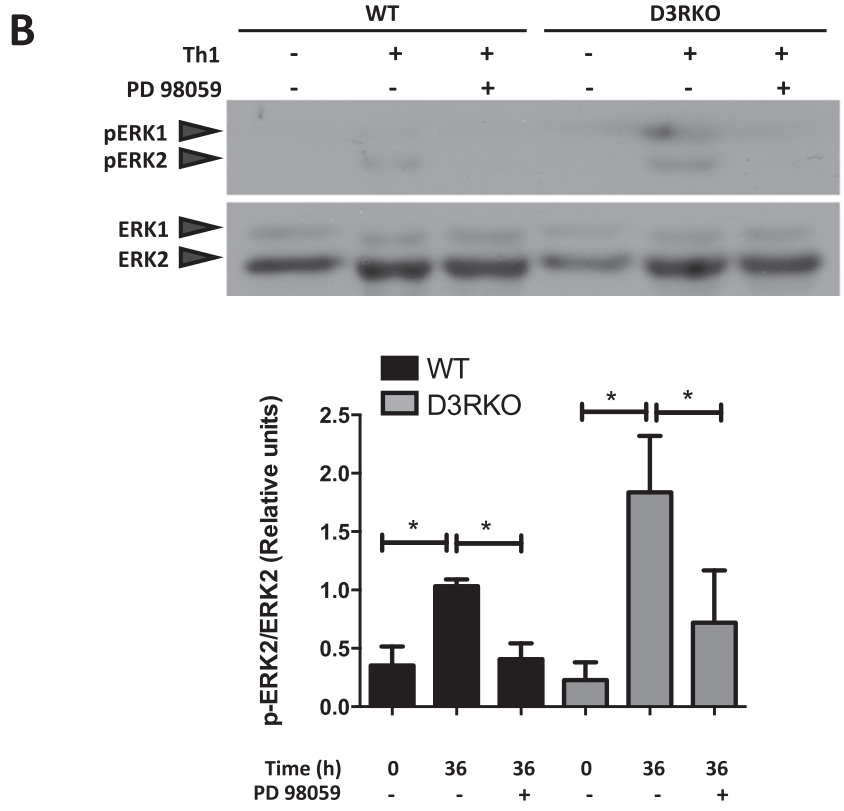


Fig. 5. Late phosphorylation of ERK2 is involved in D3R-mediated potentiation of Th1 differentiation. WT and D3RKO $CD4^{+}CD25^{-}$ naive T-cells were cultured under Th1 conditions, harvested at different time points and lysed to determinate the phosphorylation of ERK1/2 in protein extracts by *Western blot*. (A) Cells were harvested at 24, 36 and 48 h. (B) 24 h after initiated Th1 cultures the MEK1/2 inhibitor PD 98059 was added at 50 μM. Cells were recovered 36 h after initiated Th1 cultures and lysed to evaluate pERK1/2. (A and B) Representative *Western blot* images (top panels) and their quantification (bottom panels) are shown. Graphs on bottom panels show densitometric quantification from three (A) and five (B) independent experiments. Values are the ratio of pERK2/ERK2 density of each treatment normalised to the ratio of pERK2/ERK2 density associated with WT cells at time zero (A) or untreated WT control at time 36 h (B). Data represents mean ± SEM. *, $p < 0.05$ comparing different times. #, $p < 0.05$ comparing genotypes.

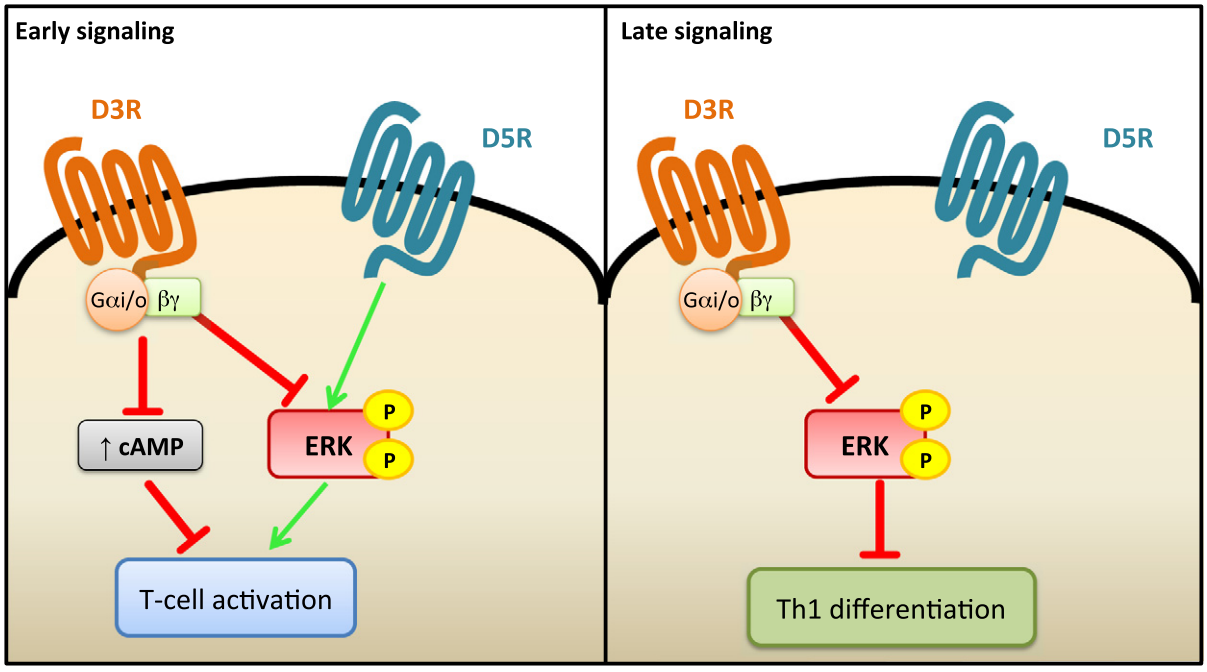


Fig. 6. Proposed model for D3R and D5R coupled signalling and regulation of down-stream functions. $CD4^+$ T-cells express both D3R and D5R. Early (0–24 h) D3R stimulation is coupled to $G\alpha_i$ activation, with a consequent decrease of intracellular cAMP levels. Thus, the stimulation of D3R during $CD4^+$ T-cell activation potentiates IL-2 secretion in a $G\alpha_i$ dependent manner. D3R-stimulation also induces a decrease in phosphorylation levels of ERK1/2, which has no consequences early in IL-2 production. Later signalling during Th1 differentiation (≈36 h) involves a wave of ERK1/2 phosphorylation that reduces the extent of Th1 differentiation, phosphorylation that is attenuated by D3R stimulation and is exacerbated in D3RKO $CD4^+$ T-cells. D5R expression in $CD4^+$ T-cells is required for early ERK1/2 phosphorylation, which correlates to efficient IL-2 secretion. Conversely, Th1 differentiation is not affected by D5R stimulation.

2012). Furthermore, WT mice bearing D5R-deficient DCs showed a mild attenuation of EAE severity, thus attributing a relevant role of D5R expressed on DCs favouring the inflammatory response associated with this mouse model of autoimmunity (Prado et al., 2012). Importantly, the difference in the extent of EAE severity observed between D5RKO mice and mice lacking D5R specifically on DCs suggests that D5R expressed in another cell population different of DCs participates in EAE favouring this $CD4^+$ T-cell mediated inflammatory response. The present study demonstrates that D5R expressed on $CD4^+$ T-cells favours T-cell activation (Fig. 3A and B). Accordingly, it is possible that D5R expressed on $CD4^+$T-cells could potentiate inflammatory responses in vivo, including the autoimmune response associated with EAE. However, the relevance of D5R expressed in $CD4^+$T-cells in vivo has not been yet addressed with genetic approaches and thereby it should be explored in future studies.

Regarding the coupling of D3R to $G\alpha_i$ and decreased cAMP observed here for murine $CD4^+$ T-cells, there is a previous study in which the coupling of D3R to adenylyl cyclase activity was analysed in human T-cells (Ilani et al., 2004). Those authors found that stimulation of human peripheral blood lymphocytes stimulated with quinpirole affected both $G\alpha_i$ and $G\alpha_s$ dependent activity. However, peripheral blood lymphocyte is a heterogeneous mix of different kinds of immune cells and quinpirole is an agonist for D2R and D3R and, thereby, the effect observed in cAMP corresponds to the integration of many effects in different kinds of cells and different DARs. Nevertheless, we found here that the stimulation of purified $CD4^+$ T-cells with the D3R-specific agonist PD 128907 decreased intracellular cAMP levels induced by forskolin and that this effect was lost in D3R-deficient $CD4^+$ T-cells (Fig. 1B). Moreover, we did not find significant differences in cAMP levels when WT or D3RKO $CD4^+$ T-cells were treated with PD 128907 in the absence of forskolin (Fig. S1A). Thus, it is likely that the effect of quinpirole on $G\alpha_s$ of peripheral blood lymphocytes observed by Ilani et al. (2004), was due to the action of the agonist in another cell population different of $CD4^+$ T-cells and/or to the action of quinpirole over D2R. Another possible explanation for the discrepancies between these studies is that D3R expressed in $CD4^+$ T-cells would be coupled to different signalling pathways in human and mouse. To answer these different possibilities, further studies would be necessary in purified human $CD4^+$T-cells treated with D3R-specific agonists or antagonists.

This work constitutes the first study involving MAPK-mediated regulation of T-cell activation and differentiation by DAR stimulation in $CD4^+$ T-cells. Importantly, a previous study performed with a human cell line derived from T-cell leukaemia has demonstrated that elevation of cAMP levels followed by PKA activation results in the inhibition of Raf-1 and consequent inhibition of ERK phosphorylation and T-cell activation (Ramstad et al., 2000). Here, we show that D3R stimulation triggers two different early signalling pathways involving the reduction of cAMP (Fig. 1B) and the inhibition of ERK2-phosphorylation (Fig. 2A and B), which have a positive effect in T-cell activation (Fig. 3A and D). Thus, the D3R-mediated potentiation of T-cell activation can be attributed to the reduction of cAMP in early signalling (Fig. 3E and F). However, the D3R-induced effect on the early inhibition of ERK2-phosphorylation has not impact in T-cell activation and it would be triggered by a signalling pathway independent of the PKA-mediated inhibition of the Raf-1–ERKs-axis. This constitutes an interesting D3R-triggered early signalling pathway for which we could not find a physiological consequence here and that should be investigated in the future. In agreement with our pharmacologic and genetic evidences demonstrating here the role of D3R in the potentiation of $CD4^+$ T-cell activation, there are previous reports in human peripheral blood mononuclear cells showing that stimulation of D2R/D3R results in increased expression of the activation markers CD69 and CD25 in the $CD4^+$T-cell population (Ilani et al., 2004; Torres et al., 2005).

A number of studies using peptides with different TCR–pMHC affinities have associated the strength and duration of ERK1/2 phosphorylation with the differentiation of $CD4^+$ T-cells toward different functional phenotypes (Constant et al., 1995; Constant and Bottomly, 1997; Tao et al., 1997). Importantly, ERK2 activation has been shown to be crucial for Th1 differentiation (Chang et al., 2012), which is in accordance with our results showing the impact of D3R-mediated signalling on ERK2 activation and consequent Th1 differentiation

(Figs. 4 and 5). Furthermore, experiments knocking down ERK1/2 with siRNA have demonstrated that Th2 differentiation is impaired and Th1 differentiation is favoured when ERK1/2-mediated signalling is decreased in naive $CD4^+$ T-cells (Tripathi et al., 2012). However, in contrast with our results a marked and sustained ERK1/2 phosphorylation has been associated with differentiation of $CD4^+$T-cells toward Th1, whereas weak and transient activation of these kinases has been related to Th2 polarization (Jorritsma et al., 2003; Yamane et al., 2005; Saraiva et al., 2009). The differences between these studies and the present work could be attributed to several experimental factors. The first important difference is that those studies have analysed T-cell differentiation in co-cultures with APCs, whereas we induced activation with anti-CD3ε and anti-CD28 mAbs and the Th1 differentiation was induced with addition of IL-12, IL-2 and anti-IL-4 mAb. The second difference is that here we analysed ERK phosphorylation during Th1 differentiation starting at 24 h and later time points, whilst in those studies the authors analysed it at times earlier than 24 h (Jorritsma et al., 2003; Saraiva et al., 2009). Moreover, in those studies an increase of IL-4 production was observed when differentiation co-cultures were treated with MEK inhibitors (Jorritsma et al., 2003; Yamane et al., 2005), whereas we observed increased IFN-γ when isolated $CD4^+$ T-cells were treated with PD 98059 (Fig. 4C). In this regard, additional cell signalling triggered by mediators coming from APCs could interfere with ERK1/2 phosphorylation in T-cells. Importantly, it has been shown that a strong early activation of ERK1/2 pathway impairs IL-4 production, whilst inhibition of early MEK activation favours an early wave of IL-4 production (Jorritsma et al., 2003; Yamane et al., 2005), which might promote further signalling on T-cells. According to this notion, since a small production of IL-4 could interfere with ERK1/2 phosphorylation, we add anti-IL-4 mAb to the differentiation culture to isolate the initial signalling without interference. All of these studies indicate that the extent of ERK1/2 phosphorylation early and late during $CD4^+$ T-cell differentiation constitute a complex system for tight regulation of T-cell fate.

With regard to the signalling pathway and physiological effect described for D5R in this study, we show that TCR-triggered early ERK2 phosphorylation and consequent T-cell activation are strongly impaired in D5R deficient $CD4^+$ T-cells (Figs. 2C and 3A). Moreover, pharmacological inhibition of MEK1/2–ERK1/2-pathway or type I DAR antagonism resulted in impaired activation of WT $CD4^+$ T-cells (Fig. 3B and C). Thus our data represents genetic and pharmacologic evidence indicating the coupling of D5R to stimulation of ERKs (Fig. 2C) and to the consequent potentiation of $CD4^+$ T-cell activation (Fig. 3A–C). These findings are in accordance with the important role of the ERK pathway in T-cell activation (Kortum et al., 2013). On the other hand, despite the critical role of ERK1/2 in Th1 differentiation (Jorritsma et al., 2003; Yamane et al., 2005; Saraiva et al., 2009; Chang et al., 2012; Tripathi et al., 2012), D5R deficiency had no impact on Th1 differentiation (Fig. 4B). According to this notion we observed that, irrespective of D5R deficiency in $CD4^+$ T-cells, the extent of Th1 differentiation was similarly affected by the MEK1/2 inhibitor PD98059 (Fig. S2B). This intriguing result could be explained by the fact that D5R deficiency affects just the TCR-induced early ERK1/2 activation without a significant effect in late ERK1/2 activation. This selective impact of D5R–ERK1/2-signalling on T-cell activation could be explained by the fact that our protocol for Th1 differentiation includes addition of exogenous recombinant IL-2 to $CD4^+$ T-cells cultures, thus avoiding any consequence of IL-2 deficiency in Th1 differentiation.

Interestingly, enhanced cAMP levels and expression of $G\alpha_s$ protein in $CD4^+$ T-cells have recently described to be required for efficient Th1 differentiation (Li et al., 2012; Yao et al., 2013). Accordingly, even when we did not detected any impact of D5R in increasing (Fig. S1B) or decreasing (Fig. 1C) intracellular cAMP levels in $CD4^+$ T-cells, we determined the effect of the type I DAR agonists SKF 81297 and SKF 38393 in Th1 differentiation. According to our genetic and pharmacologic data indicating the absence of D5R-mediated effect on cAMP production, the treatment of $CD4^+$ T-cells with SKF 81297 (Fig. S2A) or SKF 38393 (data not shown) had no effect on Th1 differentiation. Thus, these results indicate that, despite D5R-mediated signalling has an important impact on T-cell activation, it has no significant effects on Th1 differentiation. However, the impact of D5R-mediated signalling cannot be discarded in the differentiation of naive $CD4^+$ T-cells toward other $CD4^+$ T-cell effector phenotypes such as Th2 or Th17 (Nakano et al., 2009, 2011).

5. Conclusions

In summary, our findings represent genetic and pharmacologic evidence indicating the involvement of two DARs in the regulation of important signalling pathways of $CD4^+$ T-cells with consequent impact in the physiology of these cells. Whereas D3R stimulation triggers inhibition of adenylyl cyclase favouring T-cell activation, the stimulation of this receptor also evokes an inhibition of late ERK2 phosphorylation contributing to efficient Th1 differentiation. On the other hand, D5R stimulation contributes to an efficient TCR-induced early ERK1/2 phosphorylation, thus favouring T-cell activation and without impact on Th1 differentiation. This is the first study showing the signalling pathways coupled to D3R and D5R in purified murine $CD4^+$ T-cells and demonstrating the consequences of their stimulation in the physiology of these cells. Thus, this study constitutes a relevant contribution to the knowledge of the molecular mechanisms involved in the dopaminergic regulation of T-cell mediated 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.2015.05.003.

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