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RESEARCH PAPER

Comparative assessment of PDE 4 and 7 inhibitors as therapeutic agents in experimental autoimmune encephalomyelitis

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BACKGROUND AND PURPOSE

PDE4 inhibition suppresses experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS). However, side effects hinder PDE4 inhibitors clinical use. PDE7 inhibition might constitute an alternative therapeutic strategy, but few data about the anti-inflammatory potential of PDE7 inhibitors are currently available. We have used the EAE model to perform a comparative evaluation of PDE4 and PDE7 inhibition as strategies for MS treatment.

EXPERIMENTAL APPROACH

Two PDE7 inhibitors, the sulfonamide derivative BRL50481 and the recently described quinazoline compound TC3.6, were assayed to modulate EAE in SJL mice, in comparison with the well-known PDE4 inhibitor Rolipram. We evaluated clinical signs, presence of inflammatory infiltrates in CNS and anti-inflammatory markers. We also analysed the effect of these inhibitors on the inflammatory profile of spleen cells *in vitro*.

KEY RESULTS

TC3.6 prevented EAE with efficacy similar to Rolipram, while BRL50481 had no effect on the disease. Differences between both PDE7 inhibitors are discussed. Data from Rolipram and TC3.6 showed that PDE4 and PDE7 inhibition work through both common and distinct pathways. Rolipram administration caused an increase in IL-10 and IL-27 expression which was not found after TC3.6 treatment. On the other hand, both inhibitors reduced IL-17 levels, prevented infiltration in CNS and increased the expression of the T regulator cell marker Foxp3.

CONCLUSIONS AND IMPLICATIONS

These results provide new information about the effects of Rolipram on EAE, underline PDE7 inhibition as a new therapeutic target for inflammatory diseases and show the value of TC3.6 to prevent EAE, with possible consequences for new therapeutic tools in MS.

Abbreviations

BBB, blood-brain barrier; EAE, encephalomyelitis autoimmune experimental; LNC, lymph node cells; MBP, myelin basic protein; MS, multiple sclerosis; Treg, T regulator

Introduction

Experimental autoimmune encephalomyelitis (EAE) is considered as a good model of the human demyelinating disease multiple sclerosis (MS) (Constantinescu *et al.*, 2011). EAE has proved to be useful to study mechanisms underlying MS pathogenesis and to carry preclinical testing of potential therapies for the human disease (Pierson *et al.*, 2012). In fact, many of the drugs in current use have been previously assayed in this animal model (Teitelbaum *et al.*, 1971; Blaszczyk *et al.*, 1978; Yednock *et al.*, 1992). This model has also shed light on mechanisms of action of therapeutic approaches for MS initially unrelated to EAE studies (Martin-Saavedra *et al.*, 2007; 2008; Axtell *et al.*, 2010; Inoue *et al.*, 2012). Although different EAE models, depending on the antigen, species, strain or experimental condition used can determine different disease evolution (chronic or relapsing-remitting progression); some general features are shared by most EAE models. Administration of components of the CNS as antigen coupled to adjuvant to stimulate an innate immune response triggers an inflammatory reaction through activation of auto-responsive T-cells in lymph nodes and spleen able to migrate to the CNS and to cross the blood-brain barrier (BBB), where they recognize their specific antigen in the context of resident antigen presenting cells microglia or astrocytes, or immigrant macrophages or dendritic cells. These events favour an inflammatory environment with CNS injury and motor disability. Autoreactive T helper (Th) cells with Th1 (IFN-γ producers) or Th17 (IL-17 producers) phenotype are able to mediate EAE development (Chen *et al.*, 2012). Nevertheless, current trends consider IL-17 pathway as a more suitable therapeutic target over IFN-γ due to the dual role of the latter on the course of the disease (Pierson *et al.*, 2012).

Several immunomodulating cytokines like IL-4, IL-10 and IL-27 have the ability to restrain the disease (Bettelli *et al.*, 1998; Falcone *et al.*, 1998; Fitzgerald *et al.*, 2007b; Wang *et al.*, 2008). EAE development is also restricted by $Foxp3^+$ T regulator (Treg) cells (TGF-β producers) (Kohm *et al.*, 2003; McGeachy *et al.*, 2005). Sustained increase of cAMP mediates a variety of immunoregulatory signals which lead to attenuation of immune reactions and inflammation (Brudvik and Tasken, 2012). Several compounds able to increase the intracellular levels of this second messenger have long shown anti-inflammatory properties and potential to control EAE (Reder *et al.*, 1994; Genain *et al.*, 1995; Correa *et al.*, 2010). Inhibition of cAMP PDE leads to increased levels of intracellular cAMP, as these enzymes are responsible for cAMP catabolism (Mosenden and Tasken, 2011). Mainly PDE4 inhibition has been reported to improve the clinical signs of EAE (Sommer *et al.*, 1995; Martinez *et al.*, 1999; Sanchez *et al.*, 2005; Paintlia *et al.*, 2009). However, due to the difficulties to separate beneficial anti-inflammatory from adverse emetic effects of PDE4 inhibitors, efforts have been focused on finding inhibitors of other PDE that might provide therapeutic alternatives. Special interest has been directed to PDE7 inhibition as an anti-inflammatory strategy (Smith *et al.*, 2004; Giembycz and Smith, 2006; Castano *et al.*, 2009; Jerez *et al.*, 2013), and some promising results using PDE7 inhibitors in EAE have been recently reported (Redondo *et al.*, 2012a; 2012b).

In the present study, we have analysed in depth the anti-inflammatory capabilities of PDE7 inhibition, and performed a comparison between the mechanisms by which PDE4 and PDE7 inhibitors are able to ameliorate EAE. We assayed the PDE4 inhibitor Rolipram, as a well-known anti-inflammatory drug and assayed two chemically different PDE7 inhibitors drugs, the sulfonamide derivative BRL50481 (IC_{50} for PDE7A1 inhibition of 0.26 1 μM) (Smith *et al.*, 2004) and the newer quinazoline type PDE7 inhibitor TC3.6 (IC_{50} for PDE7A1 inhibition of 1.04 μM), which has shown neuroprotective properties in an *in vivo* stroke model (Redondo *et al.*, 2012c). Our results show that TC3.6, but not BRL50481, is as efficient as Rolipram in the control of EAE. In addition, we provide new information about the therapeutic action of Rolipram on EAE, and reveal that inhibition of PDE4 or PDE7 by TC3.6 act through both common and different anti-inflammatory mechanisms.

Methods

Animals, EAE induction and treatments

All experiments were conducted according to the institutional ethical and safety guidelines. SJL and C57BL/6 mice were used to perform EAE experiments and to obtain T-cells for *in vitro* assays, respectively. Murine strains were purchased from Charles River, Barcelona, Spain; SJL mice were acquired for each experiment, while C57BL-6 were breed and maintained at the animal facility of the Institution. Mice were housed in groups of 4–5 animals. Induction of EAE was performed in SJL mice by myelin basic protein (MBP) inoculation as previously described (Martin-Saavedra *et al.*, 2007). A healthy control group was established for each experiment by dispensation of the same treatment without MBP. For treatments, PDE inhibitors were daily administrated since the day of MBP inoculation (D0) by i.p. injection at the indicated dose for each case in a solution of 14% DMSO and 5% Tocrisolve (R&D Systems, Oxon, UK). Mice inoculated with 14% DMSO and 5% Tocrisolve made up the control untreated EAE group for each experiment. PDE4 inhibitor Rolipram and PDE7 inhibitor BRL50481 were purchased from Sigma-Aldrich (Madrid, Spain) and Tocris Bioscience respectively. The 3-phenyl-2,4-dithioxo-1,2,3,4-tetrahydroquinazoline PDE7 inhibitor TC3.6 was synthesized as previously described (Castano *et al.*, 2009). Clinical signs of EAE were scored according to a 0–5 scale as follows: no clinical signs, 0; loss of tail tonicity, 1; limp tail, 2; rear limbs weakness, 3; paralysis of

two limbs, 4; full paralysis of four limbs, 5. Mice were killed at day 10–15 post-inoculation by CO_2 inhalation. When any mouse reached score 4 before day 10–15 post-inoculation, it was killed in advanced. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Histopathology

Intact brains were removed from mice on day 15 post-inoculation, dissected and fixed by 10% buffered formalin. Paraffin-embedded blocks were cut into 4-μm thick sections and stained with haematoxylin and eosin and analysed by light microscopy to reveal inflammatory lesions. Quantification of perivascular infiltrates was carried out by examining four sections along the brain of each animal; sections were classified as positive or negative for infiltrates and numerical data were analysed by Fisher's test.

Cell isolation and culture

For analysis of *in vivo* PDE inhibitors effects, lymph nodes or spleens were removed from EAE animals on day 10–15 post-inoculation. Total spleen cells, lymph node cells (LNC), $CD4^+$ cell purification and CNS cell isolation were performed as described (Martin-Saavedra *et al.*, 2008). Cells from each group of EAE animals were pooled, washed and suspended in Click's medium (Click *et al.*, 1972) before *in vitro* treatment.

In vitro *T-cell phenotype polarization*

For *in vitro* T helper phenotype polarization, spleen cells from C57BL/6 mice were used. Most of the *in vitro* results obtained for C57BL/6 strain were reproduced for C3H mice to exclude possible strain specificity results. In general, the experiments were performed at 10 μM of TC3.6 and Rolipram. When other doses were used, it is indicated at the corresponding figure. Freshly isolated spleen total population or purified $CD4^+$ T-cells were *in vitro* stimulated by coated-plate anti-CD3 (YCD3-1, 50 μg mL^{-1}) (Portolés *et al.*, 1989) and soluble anti-CD28 clone 37.51 (0.5 ng mL^{-1}, eBioscience, Hatfield, UK) in Click's medium. Specific phenotype culture conditions were carried out in the presence of 10 ng mL^{-1} of IL-12 (Prepotech, London, UK) and 25 μg mL^{-1} of anti-IL-4 (11B11; ATCC HB188) for Th1 phenotype induction; or 20 ng mL^{-1} of IL-6 (eBioscience), 5 ng mL^{-1} of TGFβ (eBioscience), 25 μg mL^{-1} of anti-IL-4 (11B11; ATCC HB188) and 25 μg mL^{-1} of anti-IFN-γ (R46A2; ATCC HB170) for Th17 polarization. For Th0 samples, only anti-CD3 and anti-CD28 were used to supplement the medium. Except for Th17 induction, after 24 h of culture, 50 u mL^{-1} of IL-2 (Prepotech) were added. After a total of 3 or 7 days of culture, cells were harvested, extensively washed and restimulated as indicated for each experiment. For $CD11b^+$ cells, depletion anti-CD11b antibody (Mac-1α, Miltenyi Biotec, Madrid, Spain) was used following the manufacturer's instructions. For IL-27 neutralization, anti-IL-27p28 antibody (R&D Systems) (1 μg mL^{-1}) was added.

T-cell proliferation and soluble cytokine detection

Proliferation was measured by the colorimetric assay based on thiazolyl blue tetrazolium bromide use (Mosman, 1983). A 2×10^5 cells were split on plate-bound anti-CD3 p-96 microtitre wells supplemented with soluble anti-CD28 during 72 h. Cytokines released to the medium were quantified by ELISA. Captured and biotin-conjugated antibodies, respectively were eBio17CK15A5 and eBio17B7 (e-Bioscience) for IL-17A; Jes5-2A5 and Jes5-16E3 (BD-Bioscience, Madrid, Spain) for IL-10; R4-6A.2 and XMG1.2 BD Bioscience) for IFN-γ; and 11B11 and BVD6-24G2 (Becton-Dickinson, Madrid, Spain) for IL-4. Each sample was assayed in quadruplicate.

Measurement of mRNA expression

Retrotranscription assay and quantitative real-time PCR conditions for Foxp3, IL-17, IL-4 and IFN-γ were as previously reported (Martin-Saavedra *et al.*, 2008). Primers used for IL-27p28 were: 5′-CTCAGAGAGGGAGCAGCTGT–3′, as forward, and 5′-TCAGGTGTCATCCCAAGTGTCC-3′, as reverse. The PCR product quality was checked by a melting curve analysis for each sample and the reaction efficiencies were checked to be near 2. Each result was normalized to the housekeeping β-actin gene expression. Relative quantification of gene expression analysis was performed using the Pfaffl method (Pfaffl, 2001).

Surface and intracellular protein staining for flow cytometry analysis

After washing in staining buffer (PBS containing 0.5% BSA, 2 mM EDTA, pH 7.2), 5×10^5 cells were incubated for 15 min at 4°C with FITC-anti-CD4 purchased from BD-Bioscience. Cells were permeabilized and fixed with BD Cytofix/Cytoperm Plus (BD Biosciences) for intracellular staining with PE-anti-IL-17 (BD Biosciences), according to the manufacturer instructions.

Flow cytometry data management

Data were acquired on FACSCanto (Becton-Dickinson) flow cytometres and analysed by FACSDiva (Becton-Dickinson).

Statistics

Statistical analyses were performed with GraphPad Prism version 5.02 (GraphPad software, Inc, La Jolla, CA, USA). T-test with Welch's correction was used for unpaired data, and Fisher's test for contingency table to perivascular infiltrates quantification.

Results

PDE7 inhibition by TC3.6 prevents EAE

To perform a comparative analysis of PDE4 and PDE7 as therapeutic targets in EAE, we carried out treatments of this disease with the PDE4 inhibitor Rolipram, and the selective PDE7 inhibitors BRL50481 and TC3.6 (Figure 1A). As expected, PDE4 inhibition by Rolipram prevented the clinical signs of EAE after MBP inoculation (Figure 1B). No differences between Rolipram doses from 2.5 to 10 μg g^{-1} of body weight were found. The thioxoquinazoline derivative TC3.6, which targets PDE7, showed to be as efficient as Rolipram to control EAE at doses of 10 or 2.5 μg g^{-1}. On the contrary, BRL50481, the other PDE7 inhibitor tested, had no effect on the course

of the disease even at 10 μg g^{-1}. Accordingly, histopathological analysis by haematoxylin and eosin staining of CNS sections revealed that drastically reduced perivascular inflammatory infiltrates in Rolipram-treated animals as compared to EAE control mice, while in BRL50481 treated-mice lesions were as extensive and frequent as in the EAE control group (Figure 1C–F). Treatment with TC3.6, in turn, significantly decreased the size and number of the perivascular infiltrates found in the analysed sections, in correlation with the differences found between both PDE7 inhibitors for effects on clinical signs.

Rolipram and TC3.6 restrain T-cell activity during EAE

T-cell activity from axillary and inguinal LNC was assayed by stimulation with anti-CD3 and anti-CD28, or with antigen (MBP). In EAE animals, LNC showed a pre-activated state and therefore higher proliferative response than cells from the healthy control group to both kind of stimulus. The BRL50481-treated group did not show differences with untreated mice. However, proliferation levels of cells from Rolipram and TC3.6 groups were reduced to nearly healthy control values. Such reduction was particularly noticeable for antigen stimulated cultures (Figure 2A), suggesting a peripheral tolerance to MBP. It is well known that $Foxp3^+$ Treg cells are involved in tolerance and can help to control EAE (O'Connor and Anderton, 2008; Selvaraj and Geiger, 2008). To address if the therapeutic effect of Rolipram or TC3.6 correlates with higher Foxp3 expression, relative mRNA levels were determined by quantitative real-time PCR. LNC from animals treated with these PDE inhibitors expressed higher levels of Foxp3 mRNA than cells obtained from untreated or BRL50481-treated mice (Figure 2B). $Foxp3^+$ Treg and Th17 cell subtypes are established through mutually exclusive pathways (Bettelli *et al.*, 2006). Measurements of IL-17 expression in LNC showed reduced levels of IL-17 mRNA and soluble protein for Rolipram or TC3.6 groups as compared to the EAE group, in inverse correlation to Foxp3 mRNA levels (Figure 2C). Such result was also found for purified $CD4^+$ and for total spleen cells (not shown).

Different pathways for IL-17 decrease by PDE4 and PDE7 inhibition

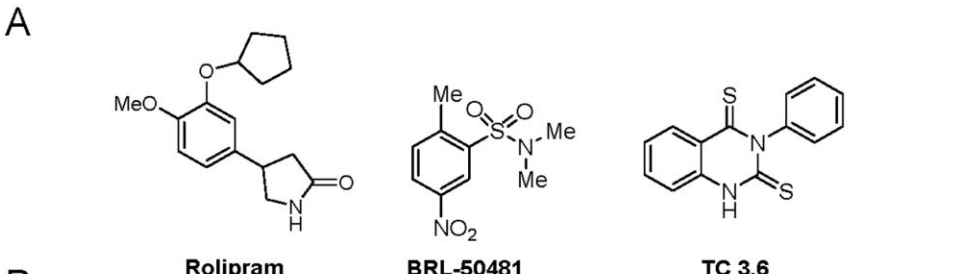
We next analysed whether the negative control of IL-17 expression by Rolipram and TC3.6 was due to a direct effect on Th17 cells. Purified $CD4^+$ cells from *naïve* mice were *in vitro* committed to Th17 phenotype by TGFβ and IL-6 in the presence of each PDE inhibitor. After Rolipram or TC3.6 removal, cells were exposed to a second round of TCR stimulation by anti-CD3. Measurements of IL-17 production showed that, in agreement with our *in vivo* results, cultures defined to Th17 in the presence of TC3.6 secreted lower levels of this cytokine than control cultures, suggesting a direct effect of TC3.6 on IL-17 producer cells. Unexpectedly, Rolipram led to higher IL-17 production than control cultures. Results were similar after 3 or 7 days of culture in the presence of PDE inhibitors previous to restimulation (Figure 3A). As IL-6 is an extremely strong inductor of IL-17 producer cells, we analysed the effect of Rolipram on anti-CD3 stimulated $CD4^+$ cultures in the absence of IL-6 and TGFβ to avoid excessive pressure towards Th17 phenotype. In these conditions, Rolipram also increased the IL-17 production in a dose-dependent way. Furthermore, the IL-17 mRNA levels were also increased by Rolipram in cells not subjected to IL-6 action (Figure 3B). Intracellular IL-17 staining also showed that Rolipram mediates an increase in the percentage of Th17 cells as an effect independent of the presence of IL-6, even if $CD4^+$ cells are subjected to Th1 conditions by IL-12 (Figure 3C, D).

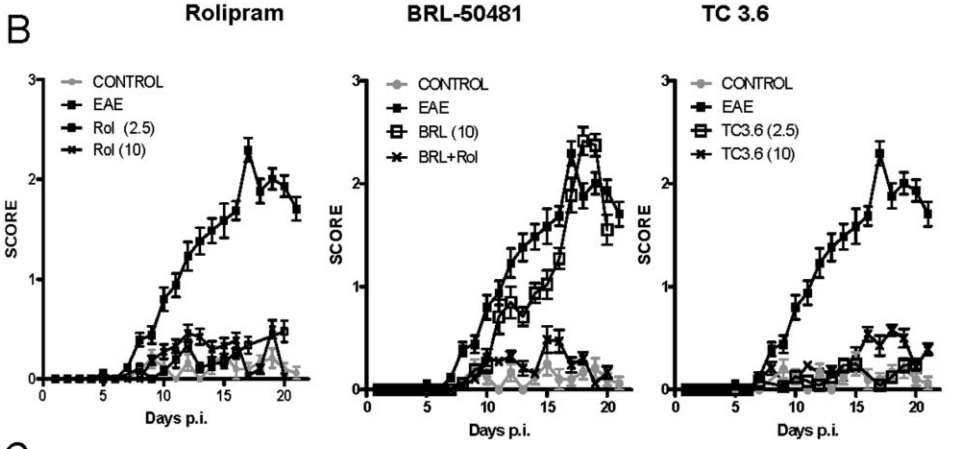
In order to explain the discrepancy between these *in vitro* results for purified $CD4^+$ cells and the decrease of IL-17 production mediated by Rolipram *in vivo*, we determined the IL-17 levels produced by a total spleen cell population exposed to Rolipram *in vitro*. In this case, neither after the first cell stimulation (Figure 4A) nor after a second round of anti-CD3 challenge (Figure 4B) was Rolipram able to increase the secretion of IL-17 at any doses assayed. On the other hand, soluble IL-17 was reduced by 50% after 3 days in the presence of Rolipram. Because activity of $CD11b^+$ cells, including macrophages, neutrophils and dendritic cell populations, has a great influence on T helper subsets of $CD4^+$ cells, we next analysed the effect of Rolipram on a spleen cell population depleted of the $CD11b^+$ fraction. Similarly to purified $CD4^+$ cells, IL-17 production was increased by Rolipram in a dose-dependent way in cultures depleted of $CD11b^+$ cells. In addition, this effect persisted even after removal of Rolipram during a second round of anti-CD3 stimulation. Interestingly, the supernatant of a total spleen population culture used as a conditioned medium to incubate purified $CD4^+$ cells blocked the increase of IL-17 expression, suggesting a soluble factor as responsible of such blockade. (Figure 4B).

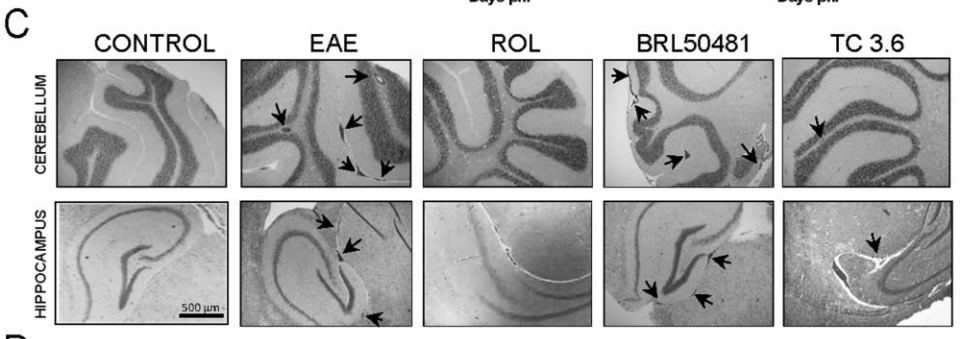
IL-27 and IL-10 levels are enhanced by Rolipram but not by TC3.6

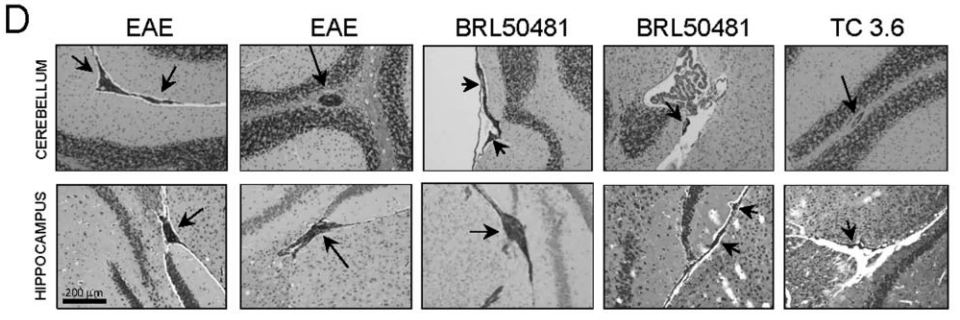
$CD11b^+$ cells, such as macrophages or dendritic cells, produce IL-27 which has a negative regulatory activity on Th17 cells (Batten *et al.*, 2006; Stumhofer *et al.*, 2006; Fitzgerald *et al.*, 2007a; Diveu *et al.*, 2009). To study the possible involvement of IL-27 in the control of the IL-17 increase mediated by Rolipram, we used an anti-IL27 antibody to neutralize this cytokine in a total spleen population culture. In these conditions, anti-IL-27 had no effect on the IL-17 levels produced by cultures treated with TC3.6. However, Rolipram was able to increase the IL-17 production, resembling the effects on purified $CD4^+$ cells (Figure 5A). This result attributes to IL-27 the prevention of the IL-17 increase mediated by Rolipram. Moreover, Rolipram led to a rise of IL-27 mRNA levels, while TC3.6 had no effect on the expression of this cytokine (Figure 5B). On the other hand, increased levels of IL-27 mRNA in spleen and spinal cord infiltrates were detected when mice were treated with Rolipram during EAE (Figure 5C).

In addition to IL-17, other cytokines produced by Th cells can be involved in the development and control of EAE. Thus, we analysed the effect of PDE4 or PDE7 inhibition during EAE on the levels of expression of IFN-γ, IL-4 and IL-10. No significant differences could be observed for IFN-γ or IL-4 expression between untreated controls and Rolipram or TC3.6 treated animals (Figure 6A, B). However, for IL-10 expression, mRNA and secreted protein levels were significantly increased in cells from animals which were treated with Rolipram, while TC3.6 treatment did not resulted in differences as compared with









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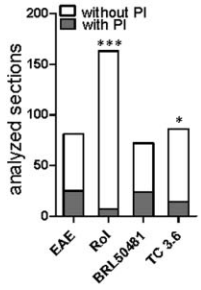
GROUP \ SECTIONS	With PI	Without PI	Total
EAE control	25	56	81
ROL	7	156	163
BRL50481	24	48	72
TC 3.6	14	72	86



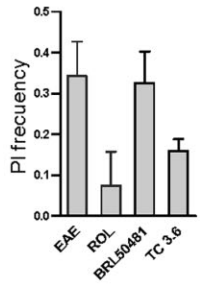


Figure 1

Effect of PDE inhibitors treatment on clinical score of MBP-EAE in SJL mice. (A): Chemical compounds used as PDE inhibitors. (B): Groups of seven mice were established by vehicle without antigen (healthy control), antigen MBP (EAE control), or MBP and the PDE inhibitor specified on the figure. Daily doses of inhibitors at µg g^{-1} of body weight are indicated in brackets. One representative experiment out of three independent assays is shown. Score values were calculated as the average of the evaluations assigned by four independent observers in blind inspection to each mouse (n = 28/group). SEM for daily values is shown. (C), (D): different magnifications of four-micrometre paraffin sections of cerebellum and hippocampus from animals killed on day 15 post-inoculation of MBP. Scale bars are indicated in the lower left micrograph for each panel. Sections were subjected to haematoxylin and eosin staining. Arrows show perivascular infiltrates. (E): Quantification of sections positive and negative for perivascular infiltrates to each group of mice. Contingency table by Fisher test was analysed by GraphPad software (***$P < 0.0001$, *$P < 0.005$). (F): Frequency of sections positive for infiltrates. Values are the average of three independent experiments and are shown as mean + SD.

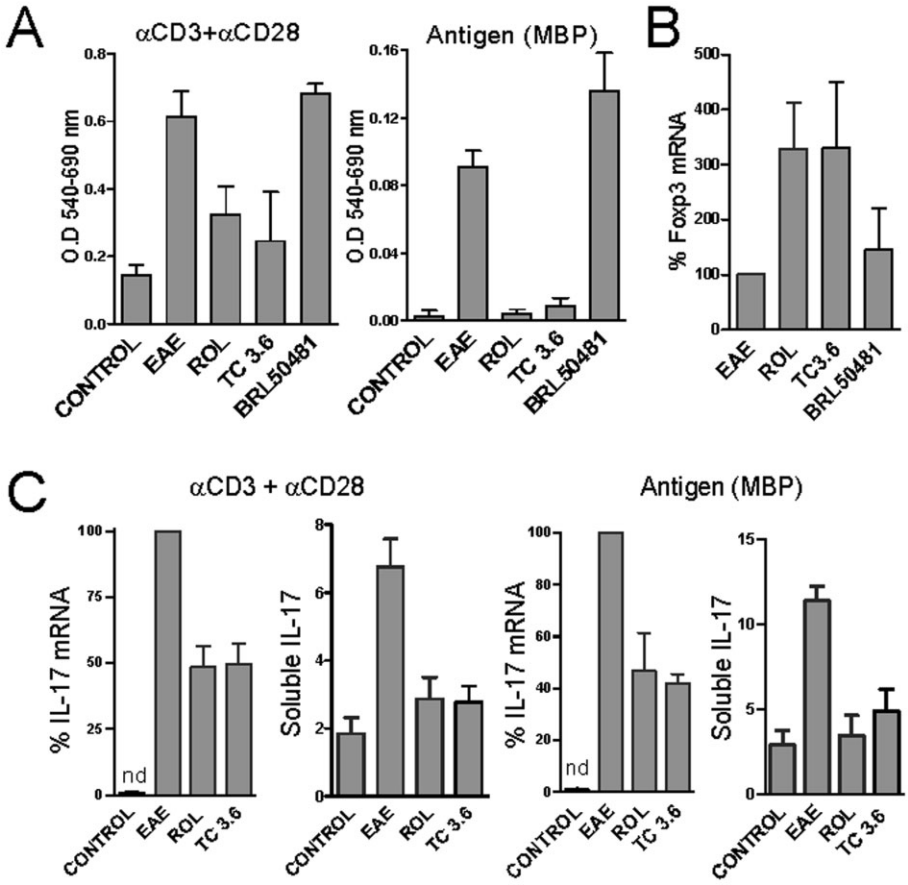


Figure 2

Influence of EAE treatment with PDE inhibitors on T-cell activity. LNC from each group of animals were pooled and stimulated by anti-CD3 and anti-CD28 (αCD3+αCD28) or by antigen (MBP) at 1x10^6 cells mL^{-1} for 72 h. Proliferation was quantified by thiazolyl blue tetrazolium bromide colorimetric method (A), Foxp3 mRNA was measured by qRT-PCR (B), and IL-17 expression was assayed by ELISA and by qRT-PCR (C). Proliferation data are given as optical density. Relative quantification of mRNA is expressed as percentages of control EAE values (considered as 100%). Soluble IL-17 results are expressed as ng mL^{-1} of protein secreted to the culture medium. Error bars are mean + SD.

cells from untreated EAE animals. A similar result was obtained for lymph nodes, spleen, pure $CD4^+$ cells and spinal cord mononuclear cells from animals killed at day 10 post-inoculation of MBP (Figure 6C).

Discussion and conclusions

The data presented here show the new PDE7 inhibitor TC3.6 as a promising anti-inflammatory tool and points to PDE7 inhibition as a new therapeutic target for inflammatory diseases. This heterocyclic quinazoline compound is as efficient as Rolipram to prevent the clinical signs of EAE. However, the previously described PDE7 inhibitor BRL50481 could not avoid the development of the disease after antigen inoculation of mice. As both PDE7 inhibitors, BRL50481 and TC3.6, have similar *in vitro* inhibition potencies on the target, with IC_{50} values of 0.26 µM for BRL50481 (Smith *et al.*, 2004) and 1 µM for TC3.6 (Castano *et al.*, 2009), their different anti-inflammatory behaviour found *in vivo* might be due to

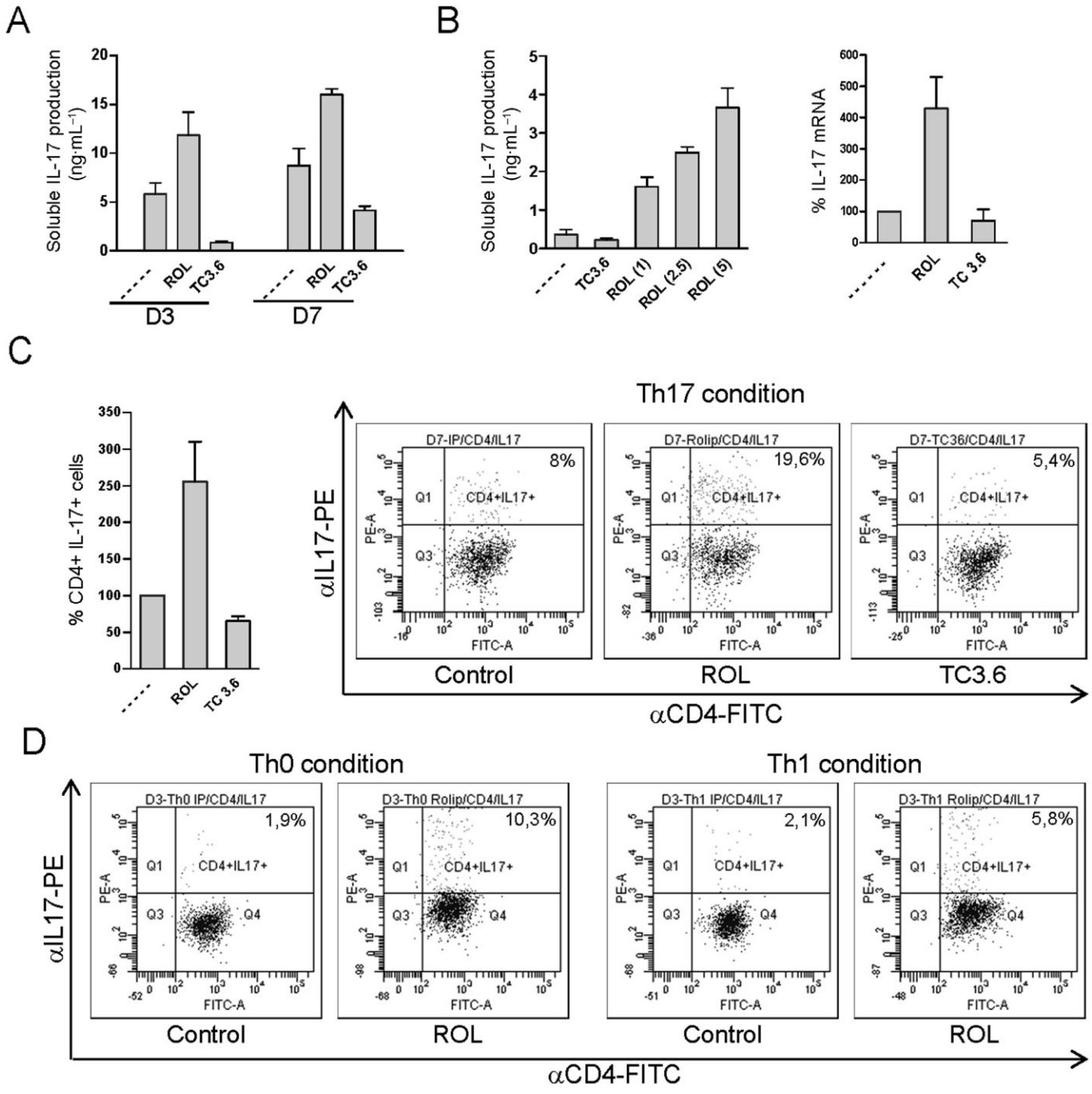


Figure 3

IL-17 expression by CD4⁺ exposed to *in vitro* PDE4 and PDE7 inhibitors treatments. CD4⁺ cells purified from *naïve* mice were cultured at Th17 (A,C), Th0 (B,D) or Th1 (D) conditions. After three (D3) or seven (D7) days of culture, PDE inhibitors were removed and cells were subjected to a second round of anti-CD3 stimulation before IL-17 expression analysis. Where it is not indicated, primary cultures were 3 days long. Soluble protein was detected by ELISA (A,B); mRNA was measured by qRT-PCR (B); and IL-17 producer cells were quantified by FACS of cells double-stained with anti-CD4-FITC and anti-IL17-PE (C,D). Results for mRNA quantification and for IL-17⁺ cells in left panel of (C) are presented as percentages of control EAE values (100%). Three independent experiments in which every sample was assayed in quadruplicate were performed for each panel. Error bars are mean + SD. FACS histograms data correspond to representative experiments and numbers on them indicate percentages of IL-17⁺.

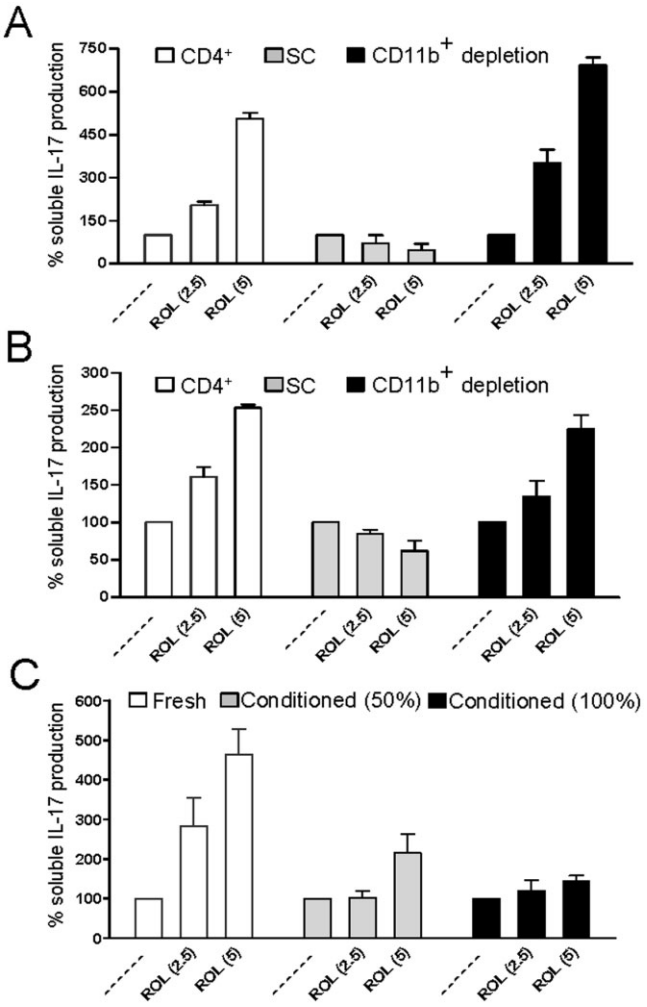


Figure 4

Influence of CD11b$^+$ cells on IL-17 expression in the presence of Rolipram. (A,B): Purified CD4$^+$ (white), total spleen cell population (grey), or spleen population depleted of the CD11b$^+$ fraction (black) were *in vitro* stimulated by anti-CD3 and anti-CD28 in the presence of Rolipram at the concentration (μM) indicated in brackets. Soluble IL-17 levels were measured by ELISA after 3 days of culture (A) or after a second round of anti-CD3 stimulation once Rolipram was removed (B). (C): Total spleen cell population was cultured during 3 days, the resulting supernatant was used as a conditioned medium to purified CD4$^+$ cell stimulation in the presence of Rolipram. Cultures were carried out in fresh Click medium (white), in Click medium one-half diluted with conditioned medium supplemented with 50% fresh FBS (grey), or in whole conditioned medium supplemented with 10% fresh FBS (black). Values are expressed as percentage of IL-17 production in respect of each control culture without Rolipram (100%). Error bars are mean + SD.

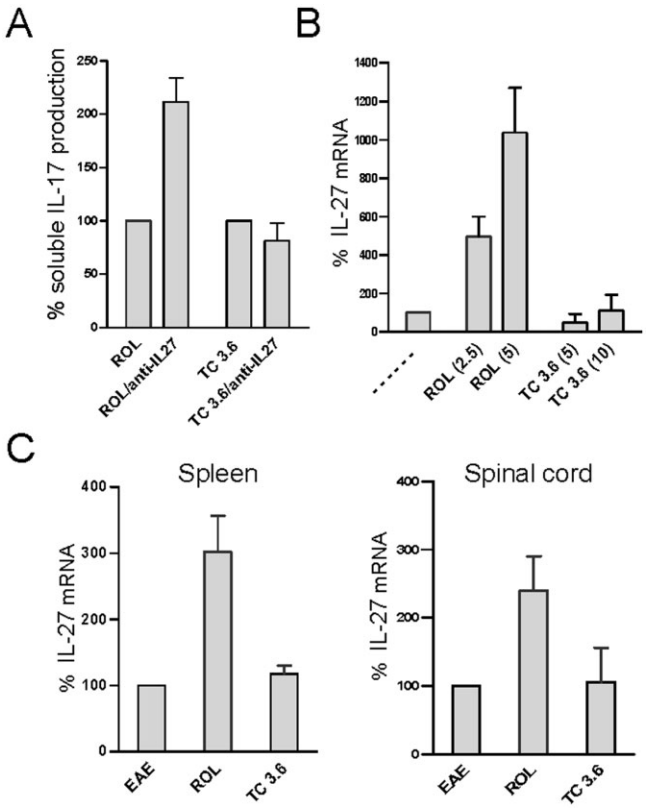


Figure 5

In vivo and *in vitro* IL-27 expression in response to Rolipram. (A): IL-17 expression under IL-27 blockade. A total spleen cell population was stimulated by anti-CD3 and anti-CD28. PDE inhibitor used and neutralizing anti-IL-27 antibody addition are indicated at the bottom. Values for samples of each PDE inhibitor are shown as percentage in respect of controls without anti-IL-27 (100%) (B): Quantification of IL-27 mRNA in cultures of spleen cells treated with Rolipram or TC3.6. Numbers in brackets indicate concentration in μM. Data are expressed as percentages in respect of control without PDE inhibitor (100%) (C): Quantification of IL-27 mRNA in spleen and spinal cord from mice subjected to EAE treatment with Rolipram or TC3.6. Values are expressed as percentages of expression in respect of untreated EAE (100%). Error bars are mean + SD for A and B, and mean + SEM with $n = 10$ for C.

differences in their physico-chemical properties. Since BRL50481 and TC3.6 have very different chemical structures, it is reasonable to think that they could have different pharmacokinetic properties, such as cell penetration ability and BBB permeation. In fact, we have strong data which support that TC3.6 is able to cross the BBB, since it has shown efficacy *in vivo* when a stroke model is used, and it is neuroprotective after i.p. administration in such model (Redondo *et al.*, 2012c). On the other hand, as far as we know, no data regarding the BBB permeation capability of BRL50481 have been published. Nevertheless, differences in ability to cross BBB could not explain all the differences found between both PDE7 inhibitors since divergence between effects of each drug spans peripheral immune events as effects on T-cell activity. Taking into account that no previous *in vivo* studies have been reported for BRL50481, the lack of peripheral effect could be due to low cell penetrability *in vivo* of this pharmacological tool.

Inhibition of PDE4 and PDE7 by Rolipram and TC3.6, respectively seem to share some mechanisms to control EAE. Both treatments reduce frequency and extent of perivascular infiltration of immune cells in CNS. Our results show that Rolipram and TC3.6 administration are also followed by common effects at primary immune response. They similarly

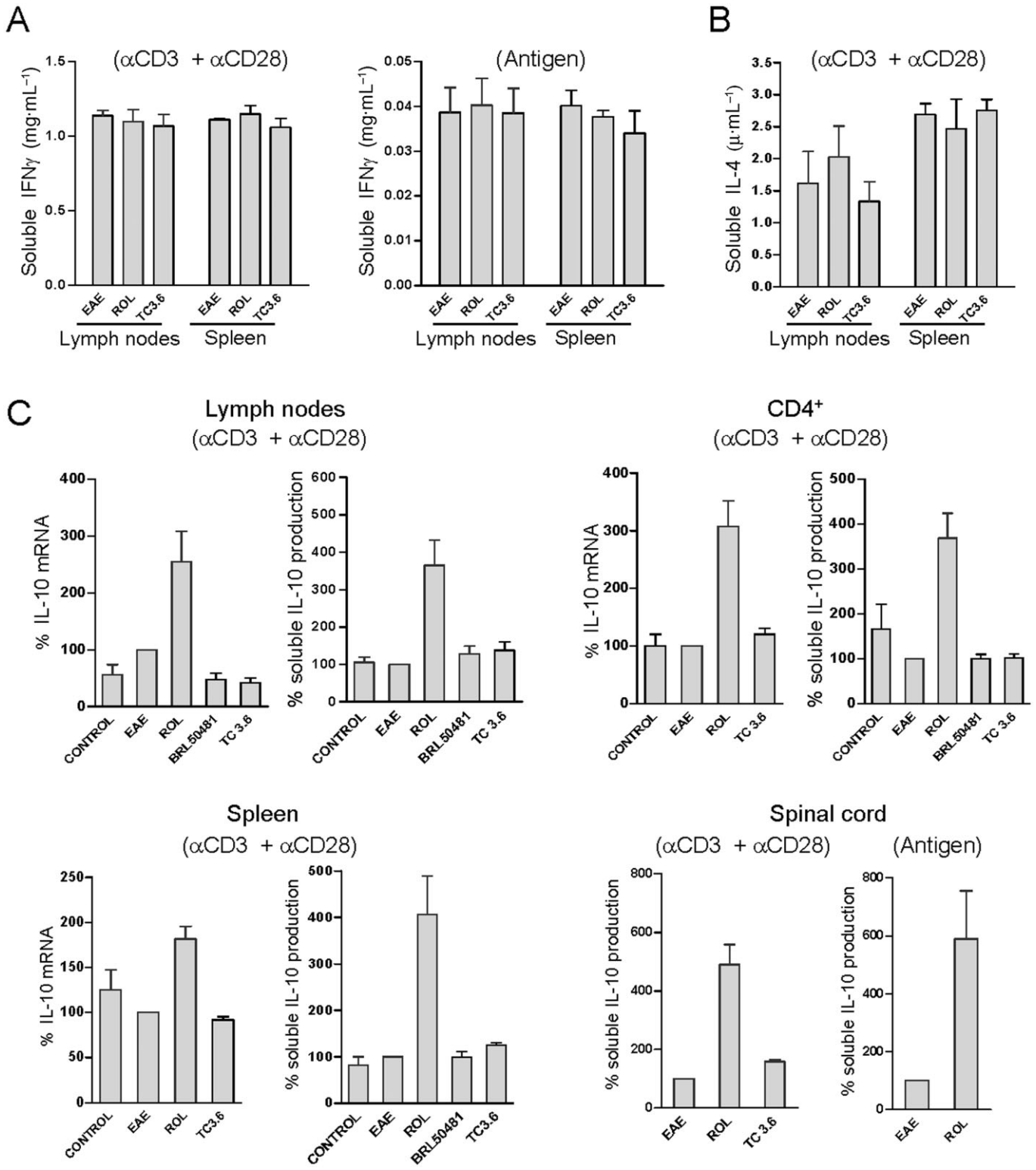


Figure 6

Expression of IFN-γ, IL-4 and IL-10 after treatment of EAE with Rolipram or TC3.6. Groups of healthy and EAE controls, and Rolipram or TC3.6 treated SJL mice were established. Mice were sacrificed at day 10 post-inoculation. Cells were obtained from spleen, lymph node and spinal cord; *in vitro* stimulated by anti-CD3 and anti-CD28 (αCD3+αCD28) or by antigen MBP (indicated on each panel). Expression of IFN-γ (A) and IL-4 (B) are expressed as absolute values of soluble protein obtained by ELISA. Expression of IL-10 (C) was measured by ELISA and by qRT-PCR and results are shown as percentages of expression in respect of EAE controls without treatment (100%). Error bars are mean + SEM with $n = 8–15$.

induce tolerance to MBP in LNC in correlation with increased levels of Foxp3 expression, suggesting involvement of induction of Treg development. Likewise, decreased production of the proinflammatory cytokine IL-17 is mediated by both PDE4 and PDE7 inhibitors. However, IL-17 decrease seems to be achieved through different pathways by each drug. As shown by our *in vitro* data, TC3.6 leads to reduction of IL-17 expression by a direct effect on purified $CD4^+$ cells, while Rolipram-mediated IL-17 decrease is only reached in the presence of $CD11b^+$ cells, indicating that this cell fraction acts as a necessary mediator for IL-17 reduction through PDE4 inhibition. In fact, Rolipram direct action on purified $CD4^+$ cells caused an increase in IL-17 production. Such effect remains after removal of the drug, suggesting that it is mediated by deviation of Th phenotype in favour of Th17. This hypothesis is supported by cytokine intracellular staining of cytokines after phenotype definition assays in the presence of the PDE4 inhibitor. This tendency towards Th17 cell differentiation provided by Rolipram overcomes even the strong influence of IL-12 to direct *naïve* $CD4^+$ cells to Th1 phenotype. Research in progress will try to elucidate the pathways through which Rolipram confers advantage to Th17 cell subtype in the absence of $CD11b^+$ cells. Nevertheless, the EAE experiments show that the net outcome of the *in vivo* treatment with Rolipram is a reduction of the IL-17 production by $CD4^+$ cells. Taking into account the aforementioned *in vitro* data, we can infer that such net anti-inflammatory effect of PDE4 inhibition might be mediated by the $CD11b^+$ fraction. Cell-to-cell contact should not be required for such intermediation since cell-free supernatant from a spleen cell culture was enough to limit the levels of IL-17, which is indicative of the involvement of a soluble factor. There are several reasons to assign this function to IL-27. First, IL-27 is one of the main negative regulators of Th17 development. Definitive studies reported that the absence of IL-27 signalling exacerbates chronic inflammation in correlation with an increased number of Th17 cells (Batten *et al.*, 2006; Stumhofer *et al.*, 2006). Second, IL-27 is mainly produced by antigen-presenting cells such as activated monocytes, macrophages and monocyte-derived dendritic cells, all of them included in the $CD11b^+$ population. Third, a role in down-modulation of Th17 cells has been previously proposed for $CD11b^+$ cells (Ehirchiou *et al.*, 2007; Valaperti *et al.*, 2008). And finally, our results regarding IL-27 neutralization in cultures treated with Rolipram revealed that this cytokine participates in restraining the IL-17 production. On the other hand, spleen cells treated with Rolipram showed an increased IL-27 expression. Consistent with these *in vitro* results, spleen cells and mononuclear cells infiltrating the spinal cord obtained from animals treated with Rolipram expressed higher levels of IL-27 mRNA. In contrast, neither *in vivo* nor *in vitro* treatment with TC3.6 induced increased IL-27 expression.

Considering all the experimental evidence, it can be outlined that the decrease of IL-17 production detected *in vivo* by PDE4 inhibition during EAE could result from the conjunction of two events: expansion of the $CD4^+Foxp3^+$Treg cell population and increase of the IL-27 production by $CD11b^+$ cells. According to the results related to IL-27, the anti-inflammatory decrease of IL-17 production in PDE7 inhibition by TC3.6 can only be ascribed to the direct effect on $CD4^+$ cells. $Foxp3^+$Treg cells are powerful suppressors of T-cell effector activity (Shevach, 2009). Besides, adaptive $Foxp3^+$ cell establishment compete in particular with Th17 phenotype since these two cell types are directed through mutually exclusive pathways. Both of them are TGFβ dependent and the decision between one or the other's fate is made by IL-6 which blocks Treg cell development and promotes Th17 phenotype definition (Bettelli *et al.*, 2006). The increase in Foxp3 expression mediated by Rolipram and TC3.6 does not correlate with decreased IFN-γ or IL-4 production. Thus, most likely, the decrease of IL-17 associated to the increase of Foxp3 expression could be attributed to a competition between $Foxp3^+$ and IL-17 producer cells rather than to suppressor activity of $Foxp3^+$ cells, which might not be enhanced enough to restrain cytokine production by other Th subtypes.

In addition to the differential effect on IL-27 mediated by Rolipram and TC3.6 treatments of EAE, the expression of the anti-inflammatory cytokine IL-10 is also only modified by Rolipram. IL-10 is an important suppressive cytokine with a pivotal role in inhibition of both antigen presentation and subsequent proinflammatory cytokine release. Its involvement in induction of T-cell tolerance with anti-inflammatory responses has been found in several organ specific autoimmune diseases, and in particular in EAE (Bettelli *et al.*, 2003). Some authors have associated the negative control of IL-17 by IL-27 to a mechanism dependent on IL-10 (Fitzgerald *et al.*, 2007b; Murugaiyan *et al.*, 2009). The influence of Rolipram on IL-10 expression has been previously reported in *in vitro* studies and in collagen-induced arthritis (Ross *et al.*, 1997; Kambayashi *et al.*, 2001). We now show a correlation between increased IL-10 production and the therapeutic effect of Rolipram on EAE.

In summary, using the heterocyclic quinazoline derivative PDE7 inhibitor TC3.6, we show here for the first time that inhibition of PDE7 during EAE reduces perivascular infiltrates in CNS, T-cell proliferation and IL17 levels, and increases Foxp3 expression. These events correlate with effective prevention of the clinical signs of the disease. Besides, we describe here new effects of Rolipram on EAE which could contribute to mediate its anti-inflammatory activity, such as reduction in IL-17 production and increase in IL-27 and IL-10 expression. Although PDE4 and PDE7 inhibition act not only through shared but also through distinct mechanisms, TC3.6 confers as efficient protection against EAE as Rolipram, emerging as an alternative to PDE4 inhibitors as anti-inflammatory tool and, hopefully, as a new therapeutic approach for the control of Th17 cell-mediated diseases. Moreover, TC3.6 has recently shown its ability to promote oligodendrocyte precursor differentiation and survival (Medina-Rodríguez *et al.*, 2013). Thus, we consider TC3.6 as a promising new drug candidate for MS pharmacological treatment, with both anti-inflammatory and neuroregenerative properties.

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Conflict of interest

Authors declare no conflict of interest.

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