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RESEARCH PAPER

Long-term suppression of EAE relapses by pharmacological impairment of epitope spreading

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BACKGROUND AND PURPOSE

Immune events sustaining dendritic cell (DC)-dependent epitope spreading (ES) are of key relevance to the development of relapses during multiple sclerosis (MS). Although no drugs are currently available to target ES, its inhibition would represent a major advancement in MS therapy. Inhibitors of the enzyme PARP-1 afford protection in animal models of MS, such as experimental autoimmune encephalomyelitis (EAE). These drugs epigenetically impair antigen presentation by DCs, but whether these drugs affect ES is unknown. Here, we investigated whether short-term treatments with these compounds would impair ES, thereby preventing EAE relapses.

EXPERIMENTAL APPROACH

We used a model of relapsing EAE in SJL mice and also adopted *in vivo* and *ex vivo* models of DC-dependent T-cell polarization. The effect of PARP-1 inhibitors on ES was evaluated at the humoral and cellular level.

KEY RESULTS

Short-term treatments with PARP-1 inhibitors during the acute phase of relapsing EAE of mice induced, at later times, more tolerogenic DCs, increased numbers of Treg cells and impairment of ES at the humoral and cellular level. These effects are followed by long-lasting reduction of relapse severity and incidence, although drug treatment had been discontinued for several weeks. PARP-1 inhibitors also induced tolerogenic DCs and increased Treg cells number and function in a model of ovalbumin immunization.

CONCLUSIONS AND IMPLICATIONS

Our data emphasize the therapeutic potential of PARP-1 inhibitors in the treatment of relapsing-remitting MS and additional ES-driven autoimmune disorders.

Abbreviations

APC, antigen-presenting cell; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; ES, epitope spreading; MS, multiple sclerosis; OVA, ovalbumin; PHE, 6-(5)-phenanthridinone; RR-MS, relapsing-remitting multiple sclerosis; Treg, regulatory T cells; SP-MS, secondary progressive multiple sclerosis

Introduction

Multiple sclerosis (MS) is one of the most common autoimmune disorders of the CNS. It typically occurs as a relapsing-remitting disease (RR-MS), followed by secondary progression (SP-MS) characterized by irreversible neurological disability (Hafler *et al.*, 2007). Unfortunately, drugs able to cure MS or halt progression of RR-MS into SP-MS are still lacking and thus

represent an unmet clinical need. It is now well appreciated that pharmacological targeting of immune events responsible for the widening of the autoimmune response within the CNS is a key strategy with realistic therapeutic potential for treatment of MS patients. In this regard, 'epitope spreading' (ES) is a dendritic cell (DC)-dependent broadening of the immune response, consisting of the continuing development of B-and T-cell clones specific for epitopes distinct from those that prompted the initial immune activation (Vanderlugt *et al.*, 1998). ES is considered of key relevance to the evolution and maintenance of autoimmune disorders, and in the context of MS, is supposed to underlie the molecular mechanisms responsible for disease relapses and progression (Vanderlugt and Miller, 2002; Bailey *et al.*, 2007). Despite the pathogenetic relevance of ES to immune-related disorders there are, at present, no compounds that can selectively target the molecular and cellular events sustaining its development.

PARP-1 is a nuclear enzyme involved in post-translational modification of chromatin-interacting proteins and epigenetic regulation of gene transcription (Kraus, 2008). In immune cells, PARP-1 activity promotes expression of inflammatory mediators mainly by regulating transcriptionally active protein complex formation and chromatin architecture (Chiarugi, 2002b; Kraus and Lis, 2003; Krishnakumar and Kraus, 2010). Recent work from our group indicates that expression of PARP-1 is induced during DC maturation, and assists in co-stimulatory antigen expression and pro-inflammatory cytokine production (Aldinucci *et al.*, 2007; Cavone *et al.*, 2011). In keeping with this, and with the key role of DCs in MS autoimmunity, several studies report that PARP-1 inhibition affords protection in different animal models of MS. Scott and colleagues showed that PARP-1 inhibitors switched the T-cell response towards an anti-inflammatory Th2 phenotype and ameliorated symptoms in mice with experimental autoimmune encephalomyelitis (EAE) (Scott *et al.*, 2001; 2004). PARP-1 inhibitors also suppress AP1 and NF-κB transcription factor activity in rats with EAE, affording protection from disease development (Chiarugi, 2002a). Further work demonstrated that PARP-1 activity was increased at the level of plaque areas of marmosets affected by EAE (Kauppinen *et al.*, 2005). Additional evidence emphasizing the pathogenetic relevance of PARP-1 to MS stems from a recent contribution indicating increased poly(ADP-ribosyl)ation in patients with SP-MS, and amelioration of disease progression by pharmacological or genetic suppression of PARP-1 in SP-EAE mice (Farez *et al.*, 2009). However, in spite of accumulating evidence indicating PARP-1 inhibitors as potential innovative drugs for MS, the molecular and cellular mechanisms underpinning their pharmacodynamic effects are not completely understood. In particular, it is also not known if short-term treatment with PARP-1 inhibitors would be enough impair DC-dependent ES, and could provide long-lasting protection from EAE/MS relapses. Answering this question would be of relevance to MS treatment, also in light of the significant clinical development of PARP-1 inhibitors (Yap *et al.*, 2011). In principle, this could occur because of impairment of DC-dependent antigen presentation and suppression of ES.

In the present study, therefore, we used different experimental approaches to assess whether short treatments with two PARP-1 inhibitors, 6-(5)-phenanthridinone (PHE) and PJ34, during the acute EAE phase, could impair DC-dependent broadening of the immune response, and by so doing, provide prolonged suppression of relapses.

Methods

Mice

All animal care and experimental procedures were performed according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC) and were approved by the Committee for Animal Care and Experimental Use of the University of Florence. 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). A total of 216 animals were used in in the experiments described here. Mouse strains were selected because of their susceptibility to relapsing-remitting EAE induction (a prototypical model used to study ES) (McMahon *et al.*, 2005) and ovalbumin (OVA) immunization (Cavone *et al.*, 2011). Female SJL/J and C57bl (6–8 weeks old) purchased from Harlan Italy Srl (Milan, Italy) were housed (5–6 per cage) with free access to food (Harlan Global Diet 2018, Harlan Laboratories, Udine, Italy) and water, and maintained on a 12 h light/dark cycle at 21°C room temperature.

Induction of EAE, clinical and neuropathological evaluation

SJL/J mice were immunized s.c. in the flanks and at the base of the tail with a total of 300 μg of proteolipid protein $(PLP)_{139–151}$ (synthesized by EspiKem Srl., University of Florence, Italy) emulsified in complete Freund's adjuvant (Sigma, Milan, Italy) and supplemented with 4 $mg{\cdot}mL^{-1}$ of *Mycobacterium tuberculosis* (strain H37Ra; Difco Laboratories, Detroit, MI, USA). Immediately thereafter and 48 h later, mice received i.p. injections of 200 ng *Pertussis* toxin (Sigma) in 100 μL PBS. At day 1 post immunization (P.I.) or beginning at the end of first acute phase, animals ($n = 10$, divided in two cages containing five animals per cage) were i.p. injected every 12 h with 30 $mg{\cdot}kg^{-1}$ PHE, 20 $mg{\cdot}kg^{-1}$ PJ34 (Sigma Aldrich, St. Louis, MO, USA) dissolved in DMSO. Immunized vehicle-treated animals ($n = 10$, divided in two cages containing five animals per cage) received the same amount of DMSO. Mice were randomly assigned to one of the three groups for treatment starting at day 1 P.I., or divided in three groups matched for disease severity for treatment beginning at the end of first acute phase. The animals were examined daily by blinded operator for disability, and were clinically graded as follows: 0, no clinical signs; 1, paralysed tail; 2, ataxia and difficulty in righting; 3, paralysis of the hind limbs and/or paresis of the forelimbs; 4, tetraparalysis; 5, moribund or death. Immunization protocol was previously optimized (Cavone *et al.*, 2011) in order to obtain a mild severity of disease during the first acute phase and subsequent relapses. At the end of the study (day 60 P.I.) or at the indicated times, mice were killed by carbon dioxide inhalation.

Treg analysis

Lymphocytes harvested from mice with EAE (five per group), pretreated or not with PHE or PJ34 (30 $mg{\cdot}kg^{-1}$, 20 $mg{\cdot}kg^{-1}$

i.p. bid, respectively) or polarized *in vitro*, were stained using mouse Treg detection Kit (eBioscience, San Diego, CA, USA) following the manufacturer's instructions. Cells were then analysed by means of seven-colour FACScalibur (BD Bioscience, San Jose, CA, USA). $CD4^+$ $CD25^+$ $Foxp3^+$ cells were considered Treg cells.

T naïve cell purification

Naïve $CD4^+CD25^-CD62L^{hi}$ T-cells were purified by immunomagnetic cell sorting using specific labelling kit from Miltenyi Biotech (Bergisch Gladbach, Germany) following the manufacturer's instructions.

Treg polarization assay

Naïve $CD4^+CD25^-CD62L^{hi}$ T-cells were purified by immunomagnetic cell sorting (130-093-227; Miltenyi Biotec). Collected cells were found to be almost exclusively (>95%) $CD4^+CD45RB^{hi}CD44^{lo}$ by flow cytometry analysis. Treg polarization was induced in the presence of antigen-presenting cell (APC). In particular, naïve T-cells were cultured for 6 days in the presence of DCs harvested from mice treated or not with PARP-1 inhibitors (five per group) for 4 days before immunization with OVA.

DCs purification

DCs were prepared by means of magnetic cell sorting using specific labelling kit from Miltenyi Biotech following the manufacturer's instructions, as described earlier (Cavone *et al.*, 2011).

Suppressive capacity of Treg cells

To assess the suppressive capacity of Treg cells present in lymph nodes of mice pretreated or not with PHE, PJ34 or vehicle alone, mixed lymphocyte populations harvested from these mice were irradiated with 600 rad in order to block their proliferative capacity. Those cells were co-cultured with a population of OVA-responsive lymphocytes. Proliferation in response to OVA re-challenge was evaluated by measuring [^{3}H]thymidine incorporation.

OVA immunization, drug administration and cell preparation

C57bl/6J mice were immunized s.c. in the flanks and at the base of the tail with a total of 200 μg of chicken egg albumin (type IV, Sigma) emulsified in complete Freund's adjuvant containing 1 mg·mL^{-1} of *M. tuberculosis*. For 4 days before immunization, animals (n = 6) were injected i.p. every 12 h with 30 mg·kg^{-1} PHE, 20 mg·kg^{-1} PJ34 dissolved in DMSO or saline solution. Immunized vehicle-treated animals received the same amount of DMSO. Mice were killed after 48 h (for DCs collection) or 8 days (T-cell proliferation assay and Treg evaluation) by carbon dioxide inhalation, and their lymph nodes digested for 15 min at 37°C in PBS containing 1 mg·mL^{-1} of Collagenase D (Roche, Milan, Italy). The cell suspension was filtered through nylon with pores of 100 μm and used for cytofluorimetric analysis or antigen-dependent proliferation assays. In some experiments, DCs and lymphocytes from lymph nodes of OVA-immunized mice treated with PHE or PJ34 were separated using magnetic-beads-conjugated antibodies (Miltenyi Biotech). The two cell populations were then mixed to evaluate DC-mediated T-cell proliferation.

Antigen-dependent T-cell proliferation

Lymphocytes obtained from lymph nodes of OVA-immunized mice pretreated or not for 4 days with PHE or PJ34 (30 or 20 mg·kg^{-1} respectively i.p., b.i.d., six animals per group) were cultured in complete RPMI in 96 wells plates (2×10^5 cells per well) and stimulated or not with OVA (50 μg·mL^{-1}). At day 3, the proliferative response was measured by [^{3}H]thymidine incorporation test as described earlier.

Determination of serum antibodies

Serum was prepared from four animals of each treatment group. Activated polystyrene ELISA plates (96 wells) were coated with 1 μg per well of recombinant $PLP_{178–191}$ peptide in pure carbonate buffer, 0.05 M (pH 9.6), and incubated at 4°C overnight. After five washes with saline containing 0.05% Tween 20, the plates were blocked using 10% fetal calf serum (FCS) in Tween saline (100 μL per well) at room temperature for 60 min. Sera diluted from 1:50 to 1:2500 were applied at 4°C for 16 h in Tween saline with 10% FCS. After five washes, 100 μL per well of alkaline-phosphatase-conjugated goat anti-mouse IgM+IgG+IgA (H + L) antibody (Southern Biotech, Birmingham, AL, USA) diluted 1:5000 in Tween/FCS saline were added. After overnight incubation and five washes, 100 μL of substrate solution consisting of 2 mg·mL^{-1} *p*-nitrophenyl phosphate in 10% diethanolamine buffer were added. The reaction was stopped after 30 min with 50 μL of 1 M NaOH, and absorbance read in a multichannel ELISA reader at 405 nm. ELISA plates, coating conditions, reagent dilutions, buffers and incubation times were tested in preliminary experiments.

Determination of DC priming ability

DCs were harvested from draining lymph nodes of EAE SJL mice (five animals per group) at indicated time point and co-cultured with naïve T-cells. The capacity of DC to induce Treg lymphocyte as well as their priming ability was evaluated by cytofluorimetric analysis and, the latter by re-challenging T lymphocytes with different antigens. At day 3, the proliferative response was measured by [^{3}H]thymidine incorporation as described above.

In vitro *induction of Treg cells*

Naive $CD4^+CD25^-CD62L^{hi}$ T-cells purified by immunomagnetic cell sorting (Miltenyi Biotec). were cultured with anti-CD3/CD28 magnetic microbeads (Invitrogen, Carlsbad, CA, USA) and TGF-β1 (Peprotech, Rocky Hill, CT, USA; 1 ng·mL^{-1}) in presence or absence of the indicated PARP-1 inhibitors for 5 days.

Data analysis

Statistical significance of differences between results was evaluated by performing ANOVA followed by Tukey's *w*-test for multiple comparisons. The *P*-value was calculated using a two-tailed test. All animals used were included in the analysis. The group sizes were selected according to previously published papers (Esposito *et al.*, 2010; Cavone *et al.*, 2011).

Results

Preventive, short treatments with PARP-1 inhibitors in RR-EAE mice reduce severity and incidence of relapses

To better clarify the therapeutic potential of PARP-1 inhibitors in MS, we first performed *in vivo* experiments aimed at evaluating the inhibitors' ability to protect against relapses in a model of RR-EAE. A widely held consensus is that a relapses are due, at least in part, to DC-dependent presentation of new autoantigens freed during the prior wave of autoimmune attack to CNS (Vanderlugt *et al.*, 1998; Vanderlugt and Miller, 2002; McMahon *et al.*, 2005). We reasoned, therefore, that the ability of PARP-1 inhibitors to induce an immature DC phenotype during the acute EAE phase (Cavone *et al.*, 2011; Cavone and Chiarugi, 2012) might compromise priming of new autoreactive T-cells and prompt immune tolerance. If this holds true, then short treatments with PARP-1 inhibitors during the acute EAE phase should afford protection from subsequent EAE relapses. To address this issue, RR-EAE SJL mice were therefore treated with the PARP-1 inhibitors PHE (30 mg·kg^{-1}) or PJ34 (20 mg·kg^{-1}) (Banasik *et al.*, 1992) with two i.p. injections per day only during the first acute phase, and then evaluated for severity and incidence of relapses. In keeping with prior work (Cavone *et al.*, 2011), treatment from day 1 to day 20 drastically reduced the neurological score of the acute phase (Figure 1A). Strikingly, we found that mice undergoing this time-restricted treatment paradigm also exhibited fewer and less severe relapses (Figure 1A and B). Next, to adopt a more clinically relevant protocol, we treated RR-EAE mice only during the first relapse and checked severity and incidence of the second one. Again, this treatment schedule not only reduced severity of neurological score during treatment, but also ameliorated severity and reduced incidence of the second relapse that occurred 2 weeks after drug suspension (Figure 1C and D).

PARP-1 inhibitors reduce ES in RR-EAE mice

The ability of PARP-1 inhibitors to reduce relapse severity and incidence when administered in a pretreatment paradigm suggested that the chemicals were able to compromise key immune events of relevance to RR-EAE evolution. Notably, ES is central to the pathogenesis of EAE relapses and necessitates

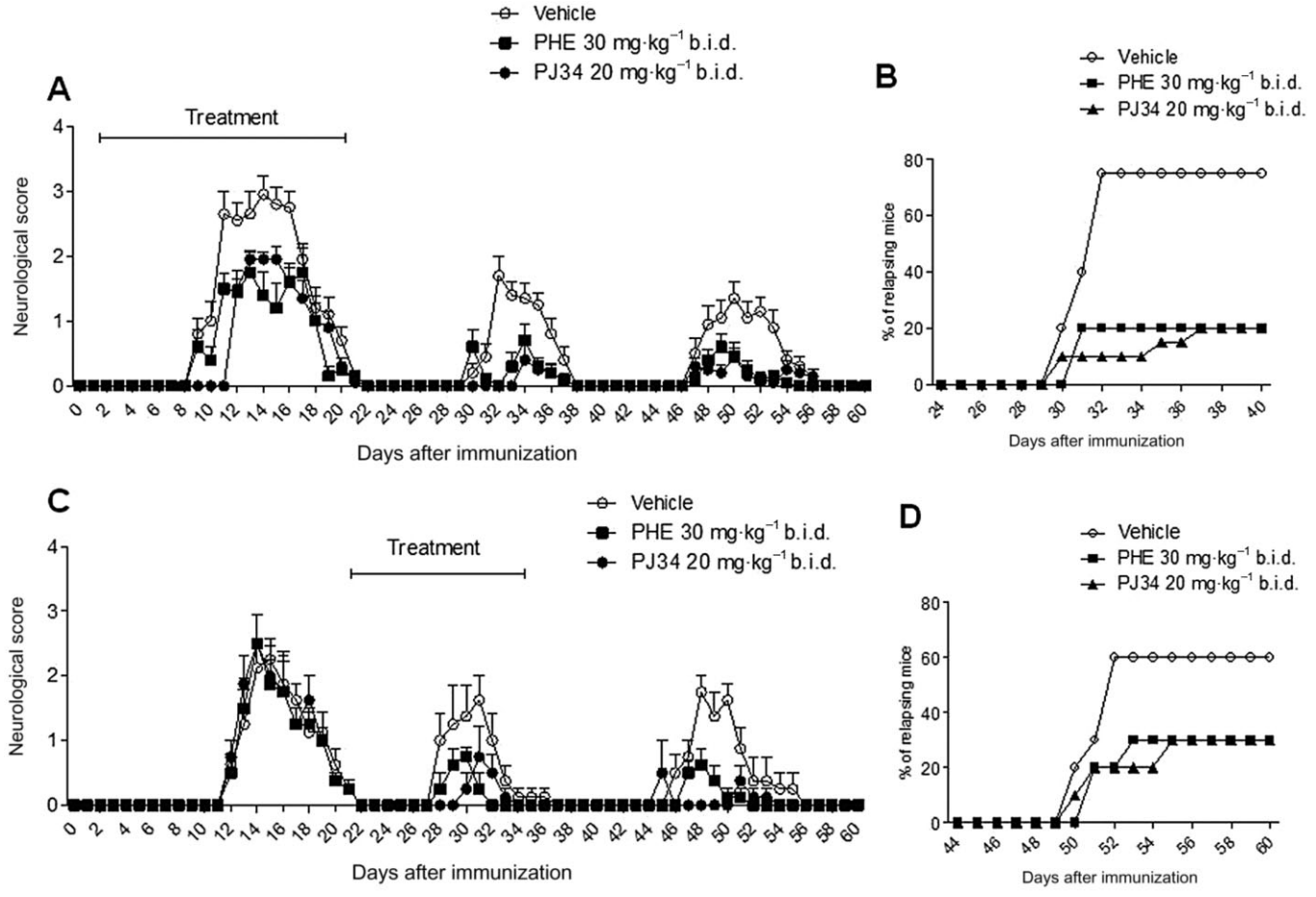

Figure 1

Short treatments with PARP-1 inhibitors (PHE, PJ34) in RR-EAE mice reduce severity and incidence of relapses. Effects of PHE or PJ34 on severity (A, C) and incidence (B, D) of relapses in RR-EAE SJL mice treated from day 1 to 20 (A, B) or from day 22 to 36 (C, D). Points represent the mean ± SEM of one representative experiment (of three).

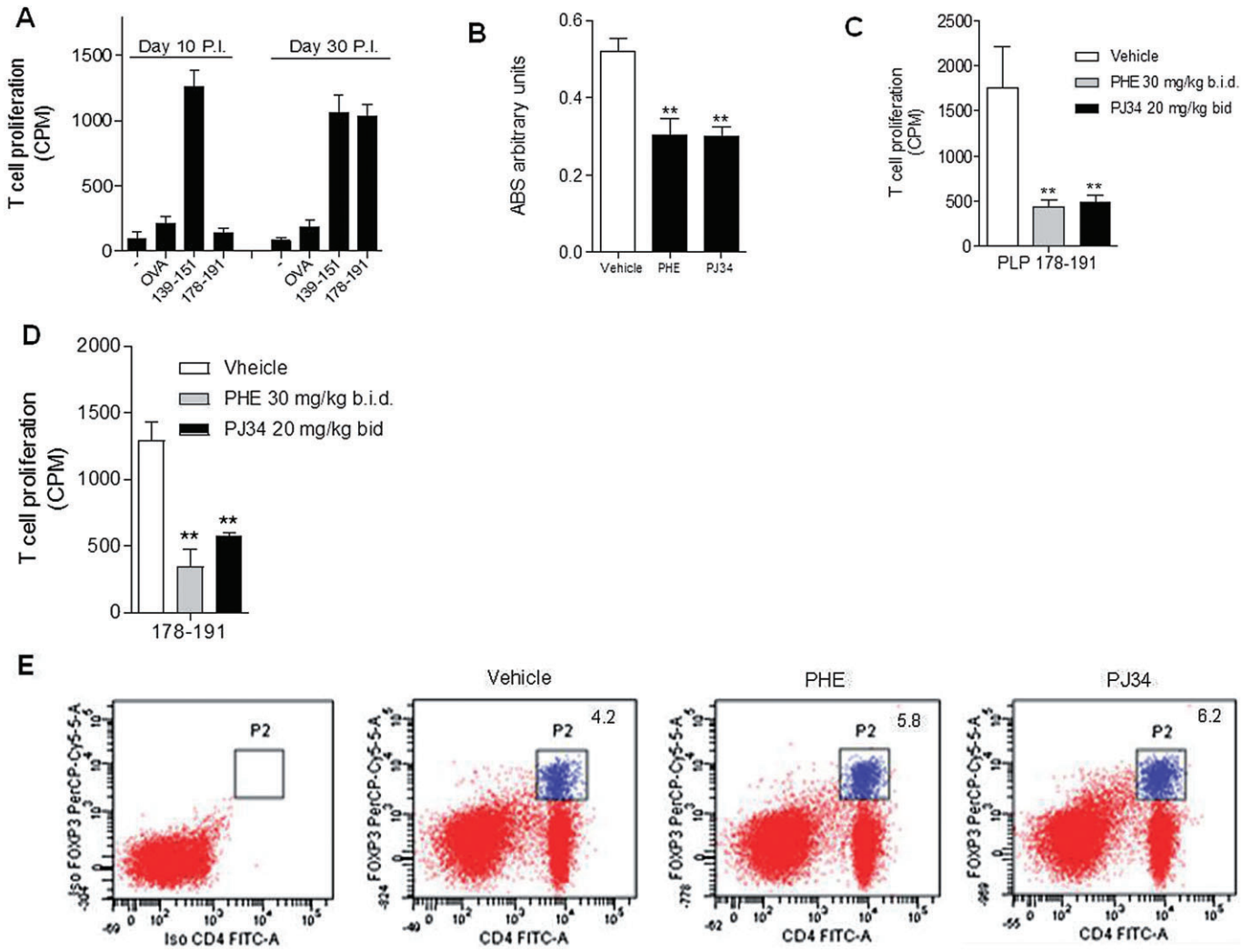

Figure 2

PARP-1 inhibitors impair ES in RR-EAE mice. Evaluation of the ability of DCs harvested at day 10 or 30 from lymph nodes of $PLP_{139–151}$-immunized mice to prompt proliferation of naïve T-cells upon re-challenge with $PLP_{139–151}$, $PLP_{178–191}$ or OVA. Evaluation of a PHE or PJ34 treatment (as shown in Figure 1A) on spreading to $PLP_{178–191}$ of the humoral (B) and cellular (C) response in $PLP_{139–151}$-immunized SJL mice at day 30 post immunization. Evaluation of the T-cell proliferating (D) or Treg-polarizing (E) ability of DCs harvested at day 30 from mice treated or not with PHE or PJ34 as shown in Figure 1A.

Bars represent the mean ± SEM of four (A–C) and two (E) experiments conducted in triplicate (A) or quadruplicate (B, C, E). In (D) a representative plot is shown. $*P < 0.05$, $**P < 0.01$ significantly different from control or vehicle. ANOVA with Tukey's *post hoc* test.

fully immunocompetent DCs to occur (McMahon *et al.*, 2005). We therefore investigated whether impairment of DC immunocompetence by PARP-1 inhibitors affected ES in RR-EAE mice. It is well established that upon the EAE acute phase in SJL mice immunized with $PLP_{139–151}$, ES prompts humoral and cellular responses to $PLP_{178–191}$ (Tuohy and Kinkel, 2000; McMahon *et al.*, 2005). In keeping with this, we found that naïve T-cells primed with DCs harvested from mouse lymph nodes 10 days after $PLP_{139–151}$ immunization (i.e. before CNS damage) proliferated upon $PLP_{139–151}$ but not $PLP_{178–191}$ or OVA re-challenge. Conversely, when DCs were harvested at day 30 (i.e. after the first wave of CNS damage), primed T-cells proliferated also upon $PLP_{178–191}$ stimulation (Figure 2A). Together, these findings confirm specificity of ES in our EAE model, and that spreading to $PLP_{178–191}$ only occurs upon the first wave of immune damage to the CNS. As shown in Figure 2B, we found that treatment of $PLP_{139–151}$-immunized mice with PARP-1 inhibitors during the acute phase (i.e. the treatment protocol shown in Figure 1A) sufficed to reduce serum antibodies against the spread epitope $PLP_{178–191}$ measured at the beginning of the first relapse (i.e. day 30). Similar findings were obtained when antigen-specific cellular responses were evaluated in these mice. Specifically, T-cells harvested at day 30 from lymph nodes of mice treated with PARP-1 inhibitors only during the acute phase were less responsive to $PLP_{178–191}$ than T-cells harvested from vehicle-injected EAE mice (Figure 2C). In an attempt to better understand the cellular basis of the impairment of the autoimmune response to $PLP_{178–191}$, we next investigated APC function of DCs harvested at day 30 from RR-EAE mice treated as shown

in Figure 1A. Of note, we found that when DCs were from mice previously treated with PARP-1 inhibitors, proliferation of lymphocytes specific for the spread epitope $PLP_{178–191}$ was lower compared with that prompted by DCs harvested from vehicle-treated mice (Figure 2D). Taken together, these findings indicate impairment of ES by PARP-1 inhibitors at the DC level. This is in consistent with the key role of PARP-1 in DC maturation and function (Aldinucci *et al.*, 2007), and with the ability of PARP-1 inhibitors to prompt immature DC phenotypes (Cavone *et al.*, 2011). This information, along with the well-established ability of immature DCs to bias T-cell proliferation towards Tregs (Tisch, 2010; Manicassamy and Pulendran, 2011), prompted us to test whether PARP-1 inhibitors promote DC-dependent Treg differentiation. We therefore investigated the Treg-polarizing ability of DCs harvested at day 30 from RR-EAE mice treated or not with PARP-1 inhibitors as shown in Figure 1A. Importantly, DCs from treated mice preferentially polarized naïve T-cells towards Tregs (Treg polarization: 3.9 ± 0.41, 5.6 ± 0.6 and 6.3 ± 0.57% for DCs from vehicle, PHE and PJ34-treated mice respectively. $P < 0.05$ vs. vehicle, ANOVA plus Tukey's *post hoc* test) (Figure 2E).

PARP inhibitors induce tolerogenic DCs in a mouse model of OVA immunization

To corroborate evidence obtained in EAE mice that PARP-1 inhibitors prompt tolerogenic DCs, we next adopted an *in vivo* mouse model of OVA immunization along with a drug treatment schedule able to specifically target APC maturation and function. To this end, mice were pretreated with PARP-1 inhibitors for 4 days and then immunized with OVA. After 8 days of drug washout, the number of Tregs in draining lymph nodes was evaluated. This protocol was designed to target DCs during maturation (occurring immediately after immunization), leaving unaffected lymphocytes proliferation, which occurs days after immunization. Remarkably, we found that Treg number increased in the lymph nodes of animals pretreated with PARP-1 inhibitors (Figure 3A and B). Then, to test whether the increased Treg number was due to a drug effect on DCs, we investigated the T-cell polarizing ability of DCs harvested 48 h after OVA immunization (i.e. 72 h after drug discontinuation). We found that DCs from mice pretreated with PARP-1 inhibitors showed a propensity to bias naïve T-cells towards Tregs (Figure 3C and D). To investigate the suppressive activity of these regulatory cells, we next evaluated the ability of irradiated, mixed lymphocyte populations harvested from OVA-immunized mice pretreated or not with PARP-1 inhibitors to reduce proliferation of OVA-specific T-cells. Interestingly, OVA-dependent lymphocyte proliferation was reduced when cells were mixed with irradiated lymphocytes harvested from PARP-treated mice (Figure 3E). In order to understand whether, in addition to a DC-dependent indirect effect, PARP-1 inhibitors also directly promote Treg differentiation, we evaluated the effect of these drugs on TGF-β-dependent Treg polarization of naïve T-cells (i.e. in the absence of DCs). Remarkably, we found that PARP-1 inhibitors reduced, rather than increased, Treg polarization (Fig 3F). Taken together, data strengthen the hypothesis that PARP-1 inhibitors promote Treg differentiation only by inducing a tolerogenic DC phenotype.

Discussion and conclusions

In this study, we provide the first evidence that PARP-1 inhibitors induce tolerogenic DCs, as well as increasing Treg polarization and function. We also originally report that these chemicals inhibit ES at both the humoral and cellular level, thereby drastically reducing incidence and severity of EAE relapses. Of note, we show that protection from EAE relapses occurs even though drug treatment is already suspended for several weeks.

A prototypical, tolerogenic phenotype of DCs has not been yet defined (Manicassamy and Pulendran, 2011), and there is no consensus about the exact qualitative and quantitative aspects leading to tolerogenic DCs (Lutz and Schuler, 2002). Still, a large body of evidence indicates that impaired expression of co-stimulatory molecules such as CD86 and reduced production of pro-inflammatory cytokines including IL-12 are prototypical features of tolerogenic DCs (Tisch, 2010; Manicassamy and Pulendran, 2011). Notably, exposure to PARP-1 inhibitors triggers all these phenotypes in DCs *in vitro* and *in vivo* (Aldinucci *et al.*, 2007; Cavone *et al.*, 2011). An additional, well-appreciated feature of tolerogenic DCs is the increased ability to polarize towards Tregs (Rutella and Lemoli, 2004; Tisch, 2010; Manicassamy and Pulendran, 2011). Remarkably, we now demonstrate that DCs harvested from EAE animals before the first relapse bias towards Tregs more efficiently, and promote antigen-specific T-cell proliferation less efficiently, if animals are treated with PARP-1 inhibitors during the acute phase (Figure 2E and D). These findings corroborate the hypothesis that pharmacological inhibition of PARP-1 compromises the autoimmune response and prompts immune tolerance. It is worth noting that these immune events precede repression of EAE relapses and are obtained for the first time by adopting a pretreatment paradigm, two features that significantly corroborate the therapeutic potential of PARP-1 inhibitors to MS treatment. Additional evidence that PARP-1 inhibitors prompt a tolerogenic DC phenotype stems from the present experiments showing that targeting DCs during OVA immunization increases Tregs number and function. Remarkably, data provide the first evidence that pharmacological treatment with PARP-1 inhibitors biases the immune response towards Tregs, an effect consistent with the increased number of Tregs in PARP-1 KO mice (Nasta *et al.*, 2010), and with the very recent evidence that PARP-1 negatively regulates Foxp3 activity (Zhang *et al.*, 2013). These studies, however, focus on PARP protein itself and do not address the role of its activity in Foxp3-dependent Treg differentiation. Our original finding that Treg polarization of naïve T-cells *in vitro* is impaired by pharmacological inhibition of PARP-1 builds upon prior work, and is in keeping with the ability of PARP-1 inhibitors to suppress T lymphocyte proliferation and function (King *et al.*, 1989; Weltin *et al.*, 1995; McNerney *et al.*, 2001).

Still, evidence that mice challenged with PARP-1 inhibitors show increased Treg number indicates that Treg suppression by these drugs is not relevant to an *in vivo* condition. This may well be due to the higher complexity of Treg-polarizing mechanisms *in vivo* than the artificial TGFβ and antiCD3/CD28 model used *in vitro*.

Promotion of Treg function by PARP-1 inhibitors, on the one hand, may well concur to the anti-inflammatory proper-

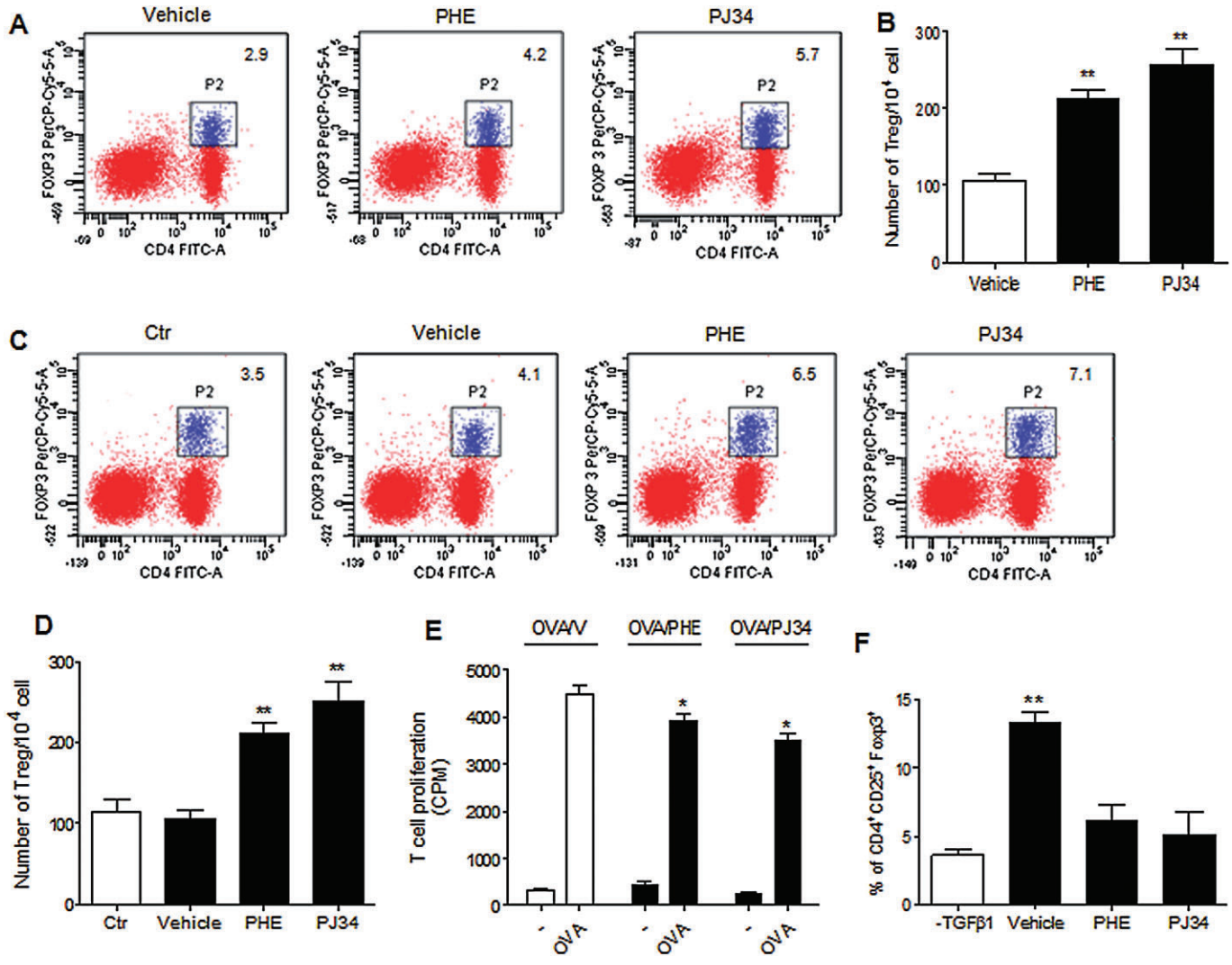

Figure 3

PARP-1 inhibitors induce tolerogenic DCs in a mouse model of OVA immunization. (A) Representative plot and quantitation (B) of FACS analysis of CD4+, CD25+, Foxp3+ cells (Treg cells) harvested 8 days after OVA immunization from draining lymph nodes of mice pretreated or not for 4 days with PHE (30 mg·kg^{-1} b.i.d.) or PJ34 (20 mg·kg^{-1} b.i.d.). (C and D) Effects of PHE and PJ34 treatment as described in (A) on the ability of DCs harvested 48 h after OVA immunization to polarize naïve spleen T-cells from syngenic mice towards Treg cells. (E) OVA-specific T-cell proliferation in the presence of an irradiated mixed lymphocyte population harvested from lymph nodes of mice pretreated or not with PHE or PJ34 as described in (A). (F) Effect of PARP-1 inhibitors on TGF-β-dependent Treg polarization of naïve T-cells. Bars represent the mean ± SEM of at least three experiments. In (A and C), representative plots are shown. *$P < 0.05$, **$P < 0.01$ significantly different from control or vehicle. ANOVA with Tukey's *post hoc* test.

ties of these chemicals shown in different models of immune activation (Jagtap and Szabo, 2005), and on the other might be harnessed for treatment of immunological disorders where increased Treg number is of therapeutic relevance (von Boehmer and Daniel, 2013).

Overall, the present study confirms the key role of poly(ADP-ribosyl)ation in molecular events sustaining DC-dependent adaptive immunity. It also furthers our understanding of the role of poly(ADP-ribosyl)ation in the complex events regulating antigen presentation during the autoimmune response. To our knowledge, this is the first experimental evidence that pharmacological treatment with PARP-1 inhibitors promotes Treg polarization and the tolerogenic response *in vitro* and *in vivo* adopting models of immune responses against both exogenous and self antigens. In light of the increasing interest on Tregs as targets of immunotherapeutic strategies in different disorders, including MS (Rutella and Lemoli, 2004), PARP-1 inhibitors emerge as potential drugs for innovative immune therapies. These compounds might, therefore, represent a significant advancement in widening the therapeutic armamentarium capable of boosting the regulatory T-cell response (Rutella and Lemoli, 2004). The considerable clinical advancement of PARP-1 inhibitors and their apparent safety profile (Rottenberg *et al.*, 2008; Yap

et al., 2011) further underscores their significance to immunomodulatory therapies for human disorders. Impairment of ES by PARP-1 inhibitors also emphasizes their therapeutic potential in various disorders such as diabetes, rheumatoid arthritis, uveitis and organ transplantation, in which spreading of the (auto)immune response to new epitopes is central to disease pathogenesis (Tuohy and Kinkel, 2000).

Conflict of interest

None.

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