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# Differential targeting of immune-cells by Pixantrone in experimental myasthenia gravis

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ABSTRACT

Pixantrone was shown to reduce the severity of clinical manifestation in experimental myasthenia gravis. In the present work we further studied its therapeutic effect. Our results demonstrate that a single administration suppressed AChR-specific immune-responses in primed rats. However, clinical symptoms could be improved only by repeated drug administrations (q7dx6 protocol—8.12 mg/kg); this treatment allowed stable serum drug levels for at least 7 days, as assessed by a functional T-cell bioassay. Pixantrone exerted strong in vitro inhibitory effect only on proliferating T-cells without impairing dendritic cell differentiation and B-cell viability. Our data further demonstrate that Pixantrone is a promising immunosuppressant drug that should be investigated in myasthenia gravis.



## 1. Introduction

MG is an antibody-mediated disorder in which autoantibodies against the acetylcholine receptors (AChR) activate the complement cascade leading to a numerical and functional loss of AChR at the neuromuscular junction (Fambrough et al., 1973; Heinemann et al., 1977; Kao and Drachman, 1977). As a consequence, neuromuscular transmission is impaired and patients complain of various degrees of muscle weakness and fatigability. Although the symptoms of MG are mediated primarily by autoantibodies, AChR-specific CD4+ T cells have a crucial role in the pathogenesis of the disease (Karachunski et al., 2000; Im et al., 2001). Indeed, effector Th1 cells secrete proinflammatory cytokines, such as IFN-γ, and are implicated in the pathogenesis of antibody-mediated autoimmune diseases, since IgG synthesis is T-cell dependent (Romagnani, 1997; Weigle and Romball, 1997). The EAMG model, characterized by the presence of CD4+ T cells and autoantibodies (IgG1 and 2 types) specific for the AChR (Christadoss et al., 2000; Baggi et al., 2012), is considered a reliable model for the human disease and is suitable for investigating the pathophysiology of MG as well as novel therapeutic strategies (Souroujon et al., 2010).

New immunosuppressant drugs are under investigation in preclinical and clinical studies to assess their efficacy on autoimmune diseases. Mitoxantrone (MTX, a synthetic anthracenedione antineoplastic compound) has been approved by the FDA for use in multiple sclerosis (Gonsette, 2007; Conway and Cohen, 2010). MTX exerts multiple effects on the immune system: suppresses proliferation of T cells, B cells, and macrophages, and impairs antigen presentation by the induction of apoptosis in APCs; moreover, experimental studies showed that also B cell function, antibody production, and transcription of proinflammatory cytokines can be impaired. Nevertheless, MTX shows several side effects, most importantly cardiac dysfunction and altered values in left ventricular ejection fraction, with an increased risk with cumulative doses above 100 mg/m$^2$ (Ghalie et al., 2002; Cohen and Mikol, 2004). Acute myeloid leukemia has been also reported (Martinelli et al., 2011).

Pixantrone (PIX) is a novel aza-anthracenedione molecule with antiblastic properties, developed to reduce the cardiotoxic effect of its analog MTX, and so far investigated only in the treatment of non-Hodgkin's lymphomas (Srokowski et al., 2011; Pettengell et al., 2012). The mechanism of action of PIX is largely unknown but is considered to be similar to that of MTX, since both drugs interact with DNA as intercalants and inhibitors of topoisomerase II (De Isabella et al., 1995).

The immunosuppressive effect of PIX has been previously investigated in EAE (Cavaletti et al., 2004; Mazzanti et al., 2005), and we

*Abbreviations:* AChR, acetylcholine receptor; TAChR, *Torpedo* acetylcholine receptor; BZ, Bortezomib; α-BTX, α-bungarotoxin; Ct, cycle threshold; EAMG, experimental autoimmune myasthenia gravis; LNCs, lymph node cells; MTX, mitoxantrone; MG, myasthenia gravis; MMF, mycophenolate mofetil; MPCs, myeloid precursor cells; PIX, Pixantrone; p.i., post immunization; RIA, Radioimmunoprecipitation Assay; SPNCs, spleen cells; [$^3$H]dT, [$^3$H]Thymidine.

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have recently demonstrated the efficacy of PIX on EAMG (16.24 mg/kg, once a week for 3 weeks q7dx3, cumulative dosage of 48.72 mg/kg) (Ubiali et al., 2008). Biological and immunological analyses confirmed that PIX reduced the severity of EAMG compared to vehicle-treated, as well as to MTX-treated EAMG rats (Ubiali et al., 2008). Therefore, the drug may have a potential role in MG treatment and hence its mechanism of action must be further investigated in EAMG in therapeutic regimens. In the current study we report new data on the efficacy of different therapeutic protocols in EAMG to identify the lowest PIX dosage still able to improve the clinical manifestation, and provide further evidences on the possible mechanisms associated to the immunosuppressive effect of this drug.

## 2. Materials and methods

### 2.1. Drug

Pixantrone (BBR2778) is an aza-anthracenedionic derivate developed by Cell Therapeutics, Inc. (Seattle, USA). Lyophilized PIX was reconstituted with sterile saline solution and added in vitro to cell cultures or administered i.v. in rats (Ubiali et al., 2008).

### 2.2. Animals

Female Lewis rats, 6–8 weeks old, were purchased from Charles River Breeding Laboratories (Calco, Italy) and kept at the animal facility of the Institute. This study has been approved by the Institute (code: IMP-03-11), and performed in accordance with the Principles of Laboratory Animal Care (European Communities Council Directive 86/609/EEC). A total of 60 animals were used for all experiments and sacrificed after deep anesthesia via carbon dioxide; for immunization and treatments animals were anesthetized with 2% isoflurane (60:40 $N_2O$:$O_2$, flow rate 0.8 l/min).

### 2.3. Antigens

AChR was purified from *Torpedo Californica* electroplax tissue (Aquatic Research Consultants, USA) by affinity chromatography on *Naja-naja siamensis* (Sigma-Aldrich) toxin coupled to Sepharose 4B (Amersham Biosciences) and analyzed on SDS–PAGE (Aharonov et al., 1977). Peptide R97–116, corresponding to region 97–116 of the rat AChR α-subunit, was synthesized by Dr. R. Longhi (C.N.R., Milan, Italy), as previously described (Baggi et al., 2004).

### 2.4. Immunization and treatment protocols

Three different single PIX dosages (4.06, 8.12, and 16.24 mg/kg) were evaluated in rats primed with 50 μg of *Torpedo* AChR (TAChR) in CFA. PIX was given 24 h after immunization and animals were sacrificed 10 days post immunization (p.i.). Lymph node cells (LNCs) were challenged in vitro with TAChR or ConA, as positive control. EAMG was induced by TAChR-immunization (50 μg TAChR in CFA); 4 weeks p.i., the animals ($n = 40$) were randomly assigned to the treatment groups described in the Results section. Treatments were performed via i.v. injections of PIX solutions or vehicle (sterile saline solution; 0.9% NaC1). The potential toxicity of repetitive drug administrations was evaluated on healthy rats ($n = 4$; protocol 16.24 mg/kg, q14dx3).

### 2.5. EAMG clinical evaluation

Evaluation of disease manifestations in EAMG rats was performed by testing muscular weakness as reported (Nessi et al., 2010). Clinical scoring was based on the presence of tremor, hunched posture, muscle strength, and fatigability. Fatigability was assessed after exercise for 30 s, using the grip strength test. Disease severity was graded as follows: grade 0, normal strength and no abnormalities; grade 1, mildly decreased activity and weak grip or cry; grade 2, clinical signs present before exercise (tremor, head down, hunched posture, weak grip); grade 3, severe clinical signs at rest, no grip, moribund; and grade 4, dead. Each animal was weighed and scored at the beginning of each experiment and twice weekly until the end of the experiment; EAMG was confirmed by Prostigmine test (i.p. injection).

### 2.6. Evaluation of PIX level in serum

Drug serum concentration was evaluated as previously reported (Cordiglieri et al., 2010). Briefly, healthy female rats were treated with one PIX i.v. injection (4.06, 8.12, 16.24 mg/kg) or PBS. Blood sampling was performed after 24 h and 7 days and sera were extracted. Sera were added, at different dilutions (1:10–1:100), to effector R97–116 specific T cells plated on CD3/CD28-coated 96-well plates for proliferation assays. In order to create a standard curve, R97–116 T cells were incubated with increasing amounts of PIX (1 pM–1 mM). After 72 h, [$^3$H] Thymidine ([$^3$H]dT, 0.5 μCi/well, PerkinElmer) incorporation was measured and PIX concentrations in animal blood were evaluated comparing the counts of serum-treated and PIX-treated R97–116 T cells.

### 2.7. Evaluation of anti rat-AChR antibody titre

Anti-rat AChR Abs were assayed in individual sera by Radioimmunoprecipitation Assay (RIA) (Lindstrom et al., 1981). Rat AChR was extracted from HD rat muscle and labeled with 2 nM [$^{125}$I] α-bungarotoxin (α-BTX, PerkinElmer). Sera from PIX- and vehicle-treated rats were incubated over-night with [$^{125}$I]-αBTX labeled rat AChR (0.5 pmol). Ab-AChR complexes were precipitated by adding an excess of rabbit anti-rat IgG (Sigma-Aldrich). Immuno-precipitates were washed twice with cold PBS plus 0.5% Triton X-100 (Carlo Erba) and [$^{125}$I]-αBTX labeling was evaluated via a γ-counter (PerkinElmer).

### 2.8. Real Time PCR

Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesized from RNA using random hexamers (Applied Biosystems) and reverse transcriptase (Invitrogen). Real-time quantitative PCR for IFN-γ, IL-10, CCR-7, CTLA-4, FoxP3, IL-6, IL-12b and β-actin was performed using Assay-on Demand Gene Expression Products (Applied Biosystems). β-Actin was used as housekeeping gene. Real-time PCR reactions were performed in duplicates using an ABI Prism 7500 FAST Real-Time PCR System (Applied Biosystems). Levels of mRNA expression for each gene were calculated using the $2^{-\Delta Ct}$ method, in which ΔCt represents the difference between cycle threshold (Ct) of the target gene and Ct of the house-keeping gene.

### 2.9. R97–116 and OVA specific T cell lines

Female Lewis rats were immunized with 50 μg of R97–116 or 50 μg of OVA (Sigma-Aldrich) in CFA (Difco Laboratories). 10 days p.i. LNs were processed into a single cell suspension. LNCs were cultured in complete RPMI 1640 medium, containing 1% Na-pyruvate, 1% non-essential aa, 1% L-glutamine, 1% penicillin–streptomycin (Euroclone Celbio), and 50 μM 2-mercaptoethanol (BDH), supplemented with 10% FBS (Gibco), and stimulated with R97–116 or OVA (5–10 μg/ml). T cell lines were maintained by repeated stimulation with the appropriate antigen every 15 days, and expanded with IL-2 (10 U/ml; Peprotech) every 3–4 days thereafter. For proliferation assays, $3 \times 10^4$ T cells were co-cultured in triplicates with $2 \times 10^5$ irradiated (3000 cGy) spleen cells (SPNCs) per well in the presence of R97–116 or OVA in RPMI medium plus 2% normal rat serum. ConA (Sigma-Aldrich) was used as positive control. Increasing concentrations of PIX (range 0.1 pM–10 μM) were added to each culture well. After 72 h [$^3$H]dT was added to each

well, later further 16 h cultures were harvested and counted on a Wallac MicroBeta TriLux counter (PerkinElmer).

### 2.10. In vitro PIX treatment of immune cells

LNs and SPN were removed from healthy and R97–116 and TAChR-primed rats after 10 p.i. and processed into single-cell suspensions; LNCs and SPNCs ($2 \times 10^5$/well) were cultured with R97–116 or TAChR and ConA in RPMI medium with 2% normal rat serum. PBMCs were isolated from the blood of unrelated human donors by centrifugation over a density gradient (Ficoll-Paque™ Plus; Amersham Biosciences), re-suspended in complete RPMI 1640 medium plus 5% human male serum type AB (Sigma-Aldrich) and plated ($2 \times 10^5$ cells/well) with PHA (10 μg/ml). Increasing concentrations of PIX (range 0.1 pM–10 μM) were added to each culture well. After 72 h, [$^3$H]dT incorporation was evaluated. Informed consents for research use of biologic material were provided by all human donors.

### 2.11. In vitro PIX treatment of myeloid precursor cells

Myeloid precursor cells (MPCs) were prepared removing tibiae and femurs from healthy female Lewis rats, and BM were processed to single-cell suspensions. After osmotic lysis in erythrolysis buffer, BM cells were seeded ($1 \times 10^6$ cells/ml) in RPMI medium for 2 h, 37 °C, 5%$CO_2$. Non-adherent cells were removed and MPCs were cultured in RPMI medium plus 10% FBS, GM-CSF and IL-4 (both at 5 ng/ml, Peprotech) for 10 days to differentiate into immature DCs (iDCs). Fresh medium with GM-CSF and IL-4 was added after 3 and 7 days of culture. Cells were exposed to increasing concentrations of PIX (10 pM–10 nM) from day 0 to 3, 0 to 7 and 3 to 10. At the end of the experiment, cell viability was evaluated by immunofluorescence, using the green-fluorimetric Tunel reaction (DeadEnd Fluorimetric Tunel kit; Promega) labeling apoptotic nuclei, according to the manufacturer's protocol. Nuclei were counter-stained with DAPI (Chemicon). As myeloid lineage marker a mouse anti-rat OX82 primary Ab (Biolegend) was used, followed by Alexa-594 conjugated goat anti-mouse Ab (Molecular Probes-Invitrogen); mouse IgG1 (Biolegend) Ab was used as negative control. Images were captured using a confocal laser-scanning microscope (D-Eclipse C1; Nikon) equipped with 40× (NA:1.30) and 60× (NA:1.40) oil objectives. ImageJ software was used for image analysis.

### 2.12. Cytofluorimetry and cytotoxic assays

MPCs from PIX-treated (one i.v. injection of PIX 4.06, 8.12, 16.24 mg/kg or 3 weekly PIX 16.24 mg/kg injections) or vehicle-treated animals, were processed to single-cell suspensions and stained with mouse anti-rat OX82 (primary Ab followed by Alexa-488 conjugated goat anti-mouse Ab (Molecular Probes-Invitrogen)). As negative control, mouse IgG1 was used at the same concentration as the primary Ab. Cells were finally labeled with 7-AAD marker (BD) to visualize dead cells. B cells were purified from LNs and spleen of healthy rats by positive selection using FITC-conjugated anti-CD45RA Ab (OX33, Serotec) and anti-FITC magnetic MicroBeads (Miltenyi Biotec). Cells were cultured in RPMI medium with 10% FBS for 3 days in the presence of PIX (10 pM–1 mM) and cell viability was evaluated using 7-AAD. All samples were acquired using a FACS vantage (BD) and gated for FSC and SSC parameters; the numbers of living and dead cells were counted relatively to a known amount of unlabeled beads appropriately gated (BD).

### 2.13. Statistical analysis

All values were expressed as means ± SE. Statistical analysis of normally distributed data was performed using Student's *t*-test for two-group analysis; two way ANOVA was used for multiple group comparisons (GraphPad Prism, CA). Statistical significance was set at $p < 0.05$.

## 3. Results

### 3.1. Improvement of EAMG by weekly administration of PIX 8.12 mg/kg, q7dx6 protocol

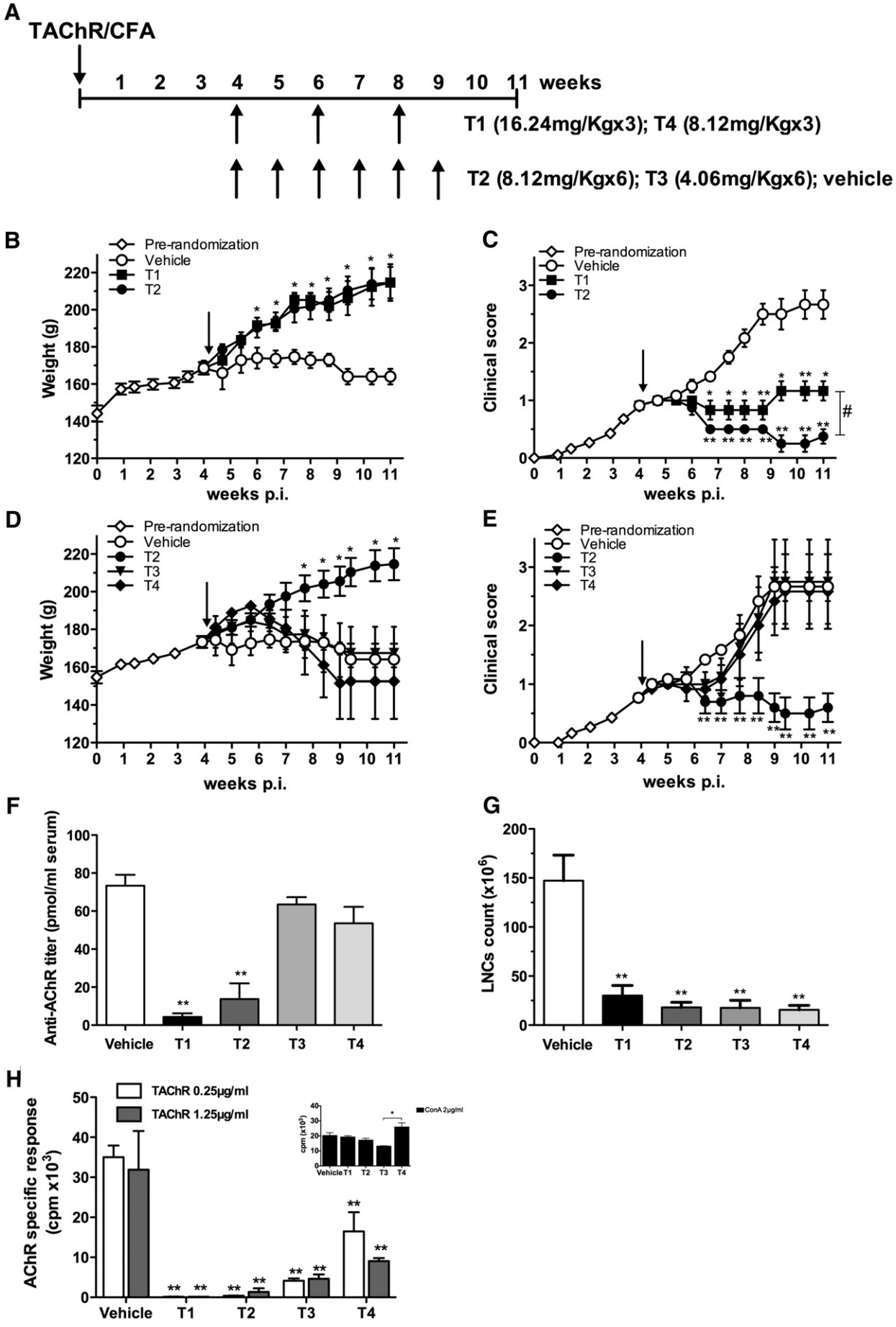
TAChR-immunized rats (n = 16) were treated with PIX according to T1 protocol (16.24 mg/kg, q14dx3, n = 5) and T2 protocol (8.12 mg/kg, q7dx6, n = 5), starting at disease onset (week = 4; treatment schedules are reported in Fig. 1A); vehicle-treated EAMG rats (q7dx6, according to T2 protocol) served as controls (n = 6). EAMG clinical manifestations (body weight, Fig. 1B, and clinical score, Fig. 1C) were significantly ameliorated by both T1 and T2 protocols, compared to vehicle-treated animals; T2 protocol also determined a further improvement in EAMG clinical score compared to T1, starting at week 7 and becoming significant at week 9 (T2 = 0.29 ± 0.27 versus T1 = 1.17 ± 0.29 mean clinical score ± SE, $p < 0.01$). Both T1 and T2 protocols correspond to a cumulative PIX dosage of 48.72 mg/kg, as the one originally reported by us (Ubiali et al., 2008), but with a different schedule.

We then investigated whether a lower cumulative PIX dosage, (24.36 mg/kg, half of the initial value) could also modulate disease manifestation. Therefore, EAMG animals (n = 24) were treated according to T3 protocol (4.06 mg/kg, q7dx6, n = 6) and T4 protocol (8.12 mg/kg, q14dx3, n = 6); EAMG animals treated with T2 protocol (n = 6) and with vehicle (n = 6) were considered as controls. T3 and T4 treatments did not clinically improve EAMG, as demonstrated by body weight (Fig. 1D) and clinical score (Fig. 1E), not different from those of vehicle-treated EAMG animals.

In order to exclude overt side effects following PIX administration, we also treated healthy rats according to T1 protocol (HD/PIX, n = 6) and monitored body weight in comparison to vehicle-treated healthy rats (HD, n = 6), assuming that a decrease of this parameter could be a possible PIX side-effect. Data indicated that PIX treatment did not induce body weight loss in HD/PIX compared to HD controls (243 ± 8.7 versus 232 ± 3.1 mean g ± SE).

In EAMG, anti-TAChR antibodies (of the IgG subtype) cross-react and recognize the rat (self) AChR located at the neuromuscular junction; hence, the level of anti-self AChR antibodies have been evaluated by the conventional Radioimmunoprecipitation Assay (RIA) in the sera of EAMG rats, collected at the end of the experiments (Fig. 1F). A significant decrease in the anti AChR-Abs titres was observed in T1-treated (4.3 ± 1.9 mean pmol/ml ± SE) and in T2-treated (13.7 ± 8.2 mean pmol/ml ± SE) animals, compared with vehicle-treated animals (73.4 ± 5.7 mean pmol/ml ± SE, $p < 0.01$), whereas animals treated according to T3 and T4 protocols showed high levels of anti AChR-Abs (63.5 ± 3.9 and 53.6 ± 8.7 mean pmol/ml ± SE, respectively for T3 and T4), values comparable to vehicle-treated controls (Fig. 1F). The decrease in pathogenic Ab levels reflected the observed modulation of EAMG symptoms (Fig. 1B–E), proving the better efficacy of the PIX dosage of 8.12 mg/kg administered every week.

To better address the action mechanism of the drug in ameliorating the ongoing disease, the effects of the different PIX treatments were evaluated on mitogen- and antigen-specific responses of single cell suspensions from lymph node (LNCs). The yield of viable mononuclear LNCs was significantly reduced, independently from the PIX protocols given to EAMG animals (T1, 30.1 ± 10.4 × $10^6$ cells; T2, 18.1 ± 5.1 × $10^6$ cells; T3, 17.5 ± 7.7 × $10^6$ cells; T4, 15.6 ± 4.5 × $10^6$ cells), compared to vehicle-treated EAMG rats (147.3 ± 25.8 × $10^6$ cells) (Fig. 1G). LNCs recovery from popliteal and inguinal LNs isolated from naïve age-matched Lewis rats was 50.1 ± 4.1 × $10^6$ cells (Supplementary data, Fig. 1A, inset). A similar reduction was not observed in the number of viable cells recovered from the spleens of PIX-treated compared with EAMG animals (data not shown).


A
TAChR/CFA
1 2 3 4 5 6 7 8 9 10 11 weeks
T1 (16.24mg/Kgx3); T4 (8.12mg/Kgx3)
T2 (8.12mg/Kgx6); T3 (4.06mg/Kgx6); vehicle
B
Pre-randomization
Vehicle
T1
T2
Weight (g)
weeks p.i.
C
Clinical score
#
D
T3
T4
E
F
Anti-AChR titer (pmol/ml serum)
Vehicle T1 T2 T3 T4
G
LNCs count (x10^6)
H
TAChR 0.25µg/ml
TAChR 1.25µg/ml
AChR specific response (cpm x10^3)
ConA 2µg/ml
cpm (x10^3)

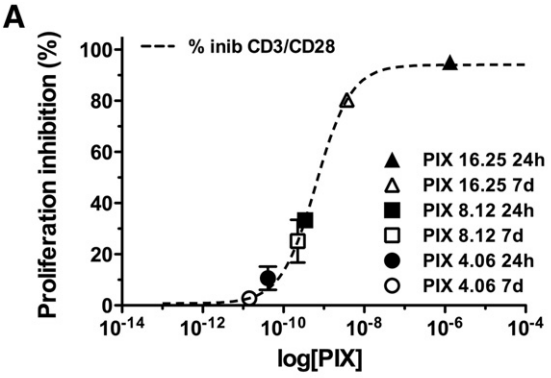
In vivo PIX treatments strongly affected TAChR-specific proliferative responses. Indeed, viable LNCs from experimental animals, stimulated ex vivo with TAChR, showed a significantly reduced proliferative response (Fig. 1H). Suppression of TAChR-specific response was almost complete in T1 and T2 treated EAMG animals: the observed residual proliferations were 0.5% (T1 protocol, for both TAChR concentrations used in vitro) and 1.1–4.2% (T2 protocol, respectively for 0.25 μg/ml and 1.25 μg/ml TAChR) of the proliferative responses from vehicle-treated EAMG animals. Although T3 and T4 protocols were not clinically effective, a significant reduction of TAChR specific responses was still observed (11.8% and 14.6% residual proliferation for T3 protocol; 47.1% and 28.3% residual proliferation for T4 protocol). On the contrary, ConA-induced LNC responses were moderately affected by PIX treatments (Fig. 1H, inset). These results suggest that, at least in our experimental conditions, in vivo PIX treatments selectively inhibit only the antigen-specific T cell responses in LNs.

To further address this hypothesis, TAChR-primed animals received a single PIX administration (4.06, 8.12, and 16.24 mg/kg) or vehicle, 24 h after immunization (n = 16, 4/group; Supplementary date, Fig. 1): all the three PIX dosages were able to significantly reduce the number of viable LNCs (panel A) as well as the proliferative responses to the priming antigen (TAChR) (panel B), but not ConA-responses (panel B, inset). The drug effect on LNCs was also studied even in naïve (healthy) rats, treated with PIX 16.24 mg/kg (HD/16.24 × 1, n = 4) or vehicle (HD/vehicle, n = 4), without observing any difference in the viable LNC numbers (HD 50.1 ± 4.1; HD/PIX 44.5 ± 2.1 × $10^6$ cells, Supplementary data, Fig. 1A, inset), further confirming the immunomodulatory effect of PIX only on stimulated immunocompetent cells.

In order to gain further data on the possible effects induced by the in vivo PIX treatments in the EAMG model, we analyzed the expression levels of selected immune-related mRNA transcripts in animals treated with T2, T3 and T4 protocols. We focused our attention to the spleen cell (SPNC) population since it was not found affected by PIX treatments, as it occurred with LNCs. mRNA targets modulated by in vivo PIX treatments were IFN-γ and IL-12b (Th1/Th17-type), IL-10 (Th2-type), CTLA4 (Treg-type), and IL-6 (Th3-type). Foxp3 (Treg-type) and CCR-7 did not show any differences (Supplementary data, Fig. 2).

### 3.2. PIX bioavailability in the periphery

EAMG course was significantly ameliorated by the T2 protocol, in which the drug has been administered i.v. at the dosage of 8.12 mg/kg for 6 times, every week. On the contrary, our data demonstrated the lack of efficacy when the same PIX dosage was administered according to T4 protocol (three administrations, every other week), or to T3 protocol (4.06 mg/kg for 6 times, every week). Hence, the level of bioactive PIX in the blood of PIX treated rats has been estimated using a functional bioassay in which animal's sera were tested for their capacity to inhibit T cell proliferation in vitro; the assay was adapted from Cordiglieri et al. (2010). Naïve animals received different doses of PIX (4.06, 8.12, and 16.24 mg/kg, single administration), and serum samples were collected from the tail vein after 24 h and 7 days; the observed inhibition values (%) of CD3/CD28 stimulated rat CD4+ T cells in the presence of serum samples have been plotted over a standard PIX dose–response inhibition curve, calculated for the same T cell line, in order to estimate PIX levels (Fig. 2). Sera from animals treated with




B

| 24 h post injection | Serum-induced proliferation inhibition | Relative PIX concentration in serum |
|---|---|---|
| PIX 4.06 | 6.4% | Log[PIX] = -10.25 ± 0.06 nM |
| PIX 8.12 | 33.3% | Log[PIX] = -9.55 ± 0.30 nM |
| PIX 16.25 | 92.5% | Log[PIX] = -7.94 ± 11.40 nM |

| 7 d post injection | Serum-induced proliferation inhibition | Relative PIX concentration in serum |
|---|---|---|
| PIX 4.06 | 2.8% | Log[PIX] = -10.83 ± 0.01 nM |
| PIX 8.12 | 25.1% | Log[PIX] = -9.68 ± 0.20 nM |
| PIX 16.25 | 80.4% | Log[PIX] = -8.82 ± 1.50 nM |

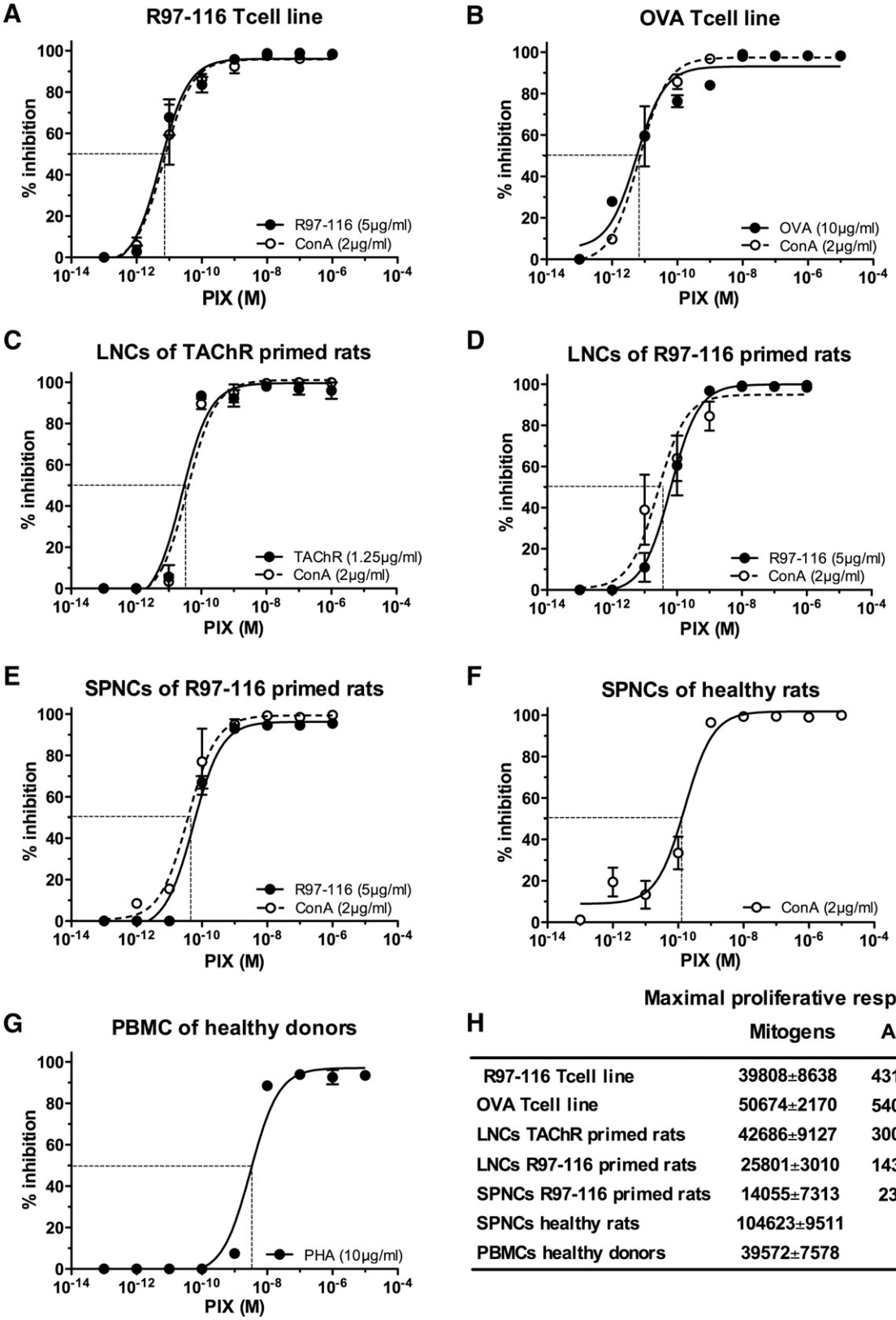
**Fig. 2.** Evaluation of PIX concentration in serum. (A) Bioassay to determine PIX concentration within the serum of animals treated with one single dose of PIX (4.06, 8.12 or 16.24 mg/kg, n = 2/group). The proliferation of R97–116 specific T cells was measured following αCD3/CD28 stimulation in presence of the indicated standard concentrations of PIX (range 0.1 pM–1 μM), or in the presence of serum extracted from treated animals 24 h or 7 days after PIX i.v. injection. R97–116 T cell reactivity was evaluated by [$^3$H]dT incorporation 3 days after stimulation. Graph represents average data ± SE from two independent experiments with triplicate measurements. Data are expressed as % of proliferation inhibition. (B) The table shows relative PIX concentrations in serum, calculated from the values of serum-induced proliferation inhibition, as measured in (A).

PIX 8.12 mg/kg showed 33.3 ± 1.5% and 25.1 ± 4.5% inhibition (samples collected after 24 h and 7 days) of the CD4+ T cell line proliferation, corresponding to a serum PIX level of 0.3 nM and 0.2 nM respectively for the two time points; calculated serum PIX levels were: 11.4 nM and 1.5 nM (24 h and 7 days for 16.24 mg/kg in vivo dose), and 0.06 nM and 0.01 nM (24 h and 7 days, 4.06 mg/kg in vivo dose). These data suggest that a serum PIX level greater than 0.2 nM should be maintained in vivo to suppress EAMG manifestations, as it occurs with the T2 but not T4 protocol.

### 3.3. PIX inhibitory dose–response effect in T cell cultures

To further elucidate the mechanisms of PIX-mediated immune suppression, we evaluated its in vitro effects on different T cell populations, exposed to PIX in the range of 0.1 pM–1 μM. First, we used rat $CD4^+$ Th1 cell lines specific for the AChR-immunodominant peptide R97–116 (Fig. 3A) or for OVA (Fig. 3B), stimulated either with the

**Fig. 1.** Comparison of different therapeutic PIX treatments on EAMG rats. (A) Schematic representation of PIX administration protocols in EAMG experiments. Arrows indicate each PIX or vehicle (0.9% NaCl) i.v. injection. (B–E) EAMG clinical course evaluation: body weight (B, D) and clinical score (C, E) of PIX-treated animals according to the administration protocols described in (A). T1 (black squares): 16.24 mg/kg, q14dx3, cumulative dosage 48.72 mg/kg; T2 (black circles): 8.12 mg/kg, q7dx6, cumulative dosage 48.72 mg/kg; T3 (black triangles): 4.06 mg/kg, q7dx6, cumulative dosage 24.36 mg/kg; T4 (black rhombs): 8.12 mg/kg, q14dx3, cumulative dosage 24.36 mg/kg; vehicle (white circles): EAMG control treated with saline solution, q7dx6; pre-randomization (white rhombs): rats before the beginning of treatments. (F) Anti-rat AChR Ab titres of EAMG rats, treated as in A–E, measured by RIA in serum samples collected at 11 weeks p.i. Anti-AChR Ab (IgG type) titres were expressed as picomoles of [$^{125}$I]-αBTX binding sites precipitated per milliliter of serum for the treatment groups. (G) Cell counts of draining (popliteal and inguinal) LNCs from EAMG animals, treated as in (A–E). (H) AChR specific responses of LNCs from EAMG animals, treated as in (A–E), and ex vivo stimulated with TAChR (0.25 μg/ml, white bars; 1.25 μg/ml, gray bars) or ConA (2 μg/ml; black bars in figure inset). Data represent average ± SE of two independent experiments. p values: $^*p < 0.05$, $^{**}p < 0.01$ compared to vehicle and ($^\#$), $p < 0.01$ compared to T1.




H

| | Maximal proliferative response to Mitogens | Antigens |
|---|---|---|
| R97-116 Tcell line | 39808±8638 | 43105±8606 |
| OVA Tcell line | 50674±2170 | 54013±1256 |
| LNCs TAChR primed rats | 42686±9127 | 30009±5939 |
| LNCs R97-116 primed rats | 25801±3010 | 14365±2962 |
| SPNCs R97-116 primed rats | 14055±7313 | 2376±1654 |
| SPNCs healthy rats | 104623±9511 | |
| PBMCs healthy donors | 39572±7578 | |

**Fig. 3.** PIX treatment inhibits actively proliferating effector T cells. (A, B) R97–116-specific (A) and OVA-specific (B) effector T-cell lines stimulated with the relevant antigen (R97–116 5 µg/ml and OVA 10 µg/ml; respectively black line and circles) or ConA (2 µg/ml; dotted line and white circles). (C–E) PIX dose–response tests performed on LNCs (C) of TAChR primed rats (n = 3), LNCs (D) and SPNCs (E) from R97–116 primed rats (n = 3), stimulated with the relevant antigen (TAChR 1.25 µg/ml and R97–116 5 µg/ml, respectively) and ConA (2 µg/ml). (F, G) SPNCs from control naïve rats (HD, n = 3, F) and human PBMCs (n = 3, G) stimulated with ConA (2 µg/ml; black line with white circles) or PHA (10 µg/ml; black line and circles) respectively. Each experiment was performed in the presence of PIX (0.1 pM–1 µM). Data represent average ± SE of three independent experiments, expressed as percentage of proliferation inhibition. (H) The table shows values of maximal proliferative responses of the different lymphocyte subpopulations of panels (A–G) stimulated with relative antigens (R97–116, OVA, TAChR) and aspecific mitogens (ConA, PHA). Data resulting from three independent experiments expressed as mean cpm ± SE.

relevant antigen or with ConA (aspecific mitogen), in the presence of PIX. The inhibitory dose–response curves were found to be similar for both T cell lines, with a calculated PIX IC50 (mean ± SE) of 5.4 ± 1.7 pM (R97–116 response) and 6.7 ± 1.6 pM (ConA response) for R97–116 T cell line; 5.6 ± 1.9 pM (OVA response) and 6.7 ± 2.0 pM (ConA response) for OVA T cell line.

Next, we investigated PIX effect on mononuclear cell cultures from draining LNs and SPNs of TAChR or R97–116 primed animals, stimulated with the relevant antigen or with ConA, and exposed to increasing doses of PIX. Calculated PIX IC50 (mean ± SE) for LNCs of TAChR-primed animals were 25 ± 3.3 pM (TAChR response) and 37 ± 2.8 pM (ConA response) (Fig. 3C). Similar values were obtained from R97–116 primed animals: PIX IC50 was 63.3 ± 1.9 pM (R97–116 response) and 25.7 ± 3.0 pM (ConA response) (Fig. 3D). PIX IC50 for SPNC cultures was 54.3 ± 1.7 pM (R97–116 response) and 37.4 ± 1.7 pM (ConA response) (Fig. 3E). Maximal proliferative responses in absence of PIX treatment are reported in Fig. 3H.

Our data show that the amount of PIX required to exert inhibitory effect on LNC or SPNC cultures was 5–12 fold greater than that required to suppress responses of antigen specific T cell lines.

To complete our analysis, we evaluated PIX effect on SPNCs from naïve Lewis rats and on PBMCs from healthy human donors, stimulated with ConA and PHA, respectively (Fig. 3F–G). PIX IC50 (mean ± SE) was 679.5 ± 2.2 pM (ConA-stimulated rat SPNCs) and 3362.0 ± 3.4 pM (PHA stimulated PBMCs). Again, a further increase in the PIX concentration is required by naïve rat SPNCs (100 fold), and even more by human PBMCs (1000 fold), as compared with those derived from antigen specific T cells.

### *3.4. PIX inhibitory effect on culture and differentiation of myeloid precursor cells*

Because of the pivotal role of DCs in the antigen processing and presentation to T cells, we evaluated the effect of PIX on rat myeloid precursor cells (MPCs) derived from BM during the in vitro differentiation to iDCs. MPCs from naïve Lewis rats were treated with PIX 0.01 nM (twice the IC50 value for T cells), 0.1, 1, and 10 nM, or normal culture medium, during the differentiation process into iDCs, and at different time points (Fig. 4A); cell viability was evaluated at the end of the differentiation period (day = 10) by immunofluorescence (Tunel). iDC viability was not affected by PIX at 0.01, 0.1, and 1 nM concentrations (Fig. 4B); a significant reduction in cell viability was observed when the differentiating MPCs were treated with the highest dose of PIX (10 nM), independently from the different exposure time (PIX-A, -B, -C), leading to only 10–15% cell loss. These data indicate also that PIX is effective when given either in the first days of MPC differentiation (PIX-A, 0–3 days) or during the final maturation phase to iDCs (PIX-C) (Fig. 4B). Morphological analysis through confocal microscopy revealed a blockade of iDC differentiation, as highlighted by the generalized round shape of the cells and the absence of cellular extensions among MPCs treated with PIX 10 nM (Fig. 4C); this was not observed during treatment with PIX 0.01 nM (Fig. 4C), or with intermediate concentrations, conditions that, on the contrary, are effective in inhibiting T cell proliferation. The potential cytotoxicity of PIX was also evaluated on $OX33^+$ B cell, immunomagnetically selected from LNs and SPN of healthy rats. $OX33^+$ was treated in vitro with PIX (range 0.1 pM–1 mM) for 3 days, and cell viability was measured via cytofluorimetry. Low doses of PIX (0.1 pM–10 nM) did not show any effect on the number of viable $OX33^+$ cells, while 20% cell death was detectable only with 100 nM–1 mM PIX (Fig. 4D).

Moreover, the effect of in vivo PIX administration was evaluated on MPCs derived from BM of naïve animals (HD), to assess whether PIX induced a generalized myelotoxicity. Animals received a single PIX dose (4.06, 8.12, 16.24 mg/kg) or three PIX doses (16.24 mg/kg, every week), and the number of total MPCs and $OX82^+$ MPCs was evaluated (Fig. 4E). We did not observe any difference among PIX- and vehicle-treated animals, independently from the PIX dosage. The cytofluorimetric data were confirmed by Trypan blue viability assay (data not shown).

Hence, low PIX levels are effective in blocking effector T cell activation and proliferation (Fig. 3 and Supplementary data, Fig. 1), without affecting myeloid cells and B cells, at least in our experimental conditions (Fig. 4).

## 4. Discussion

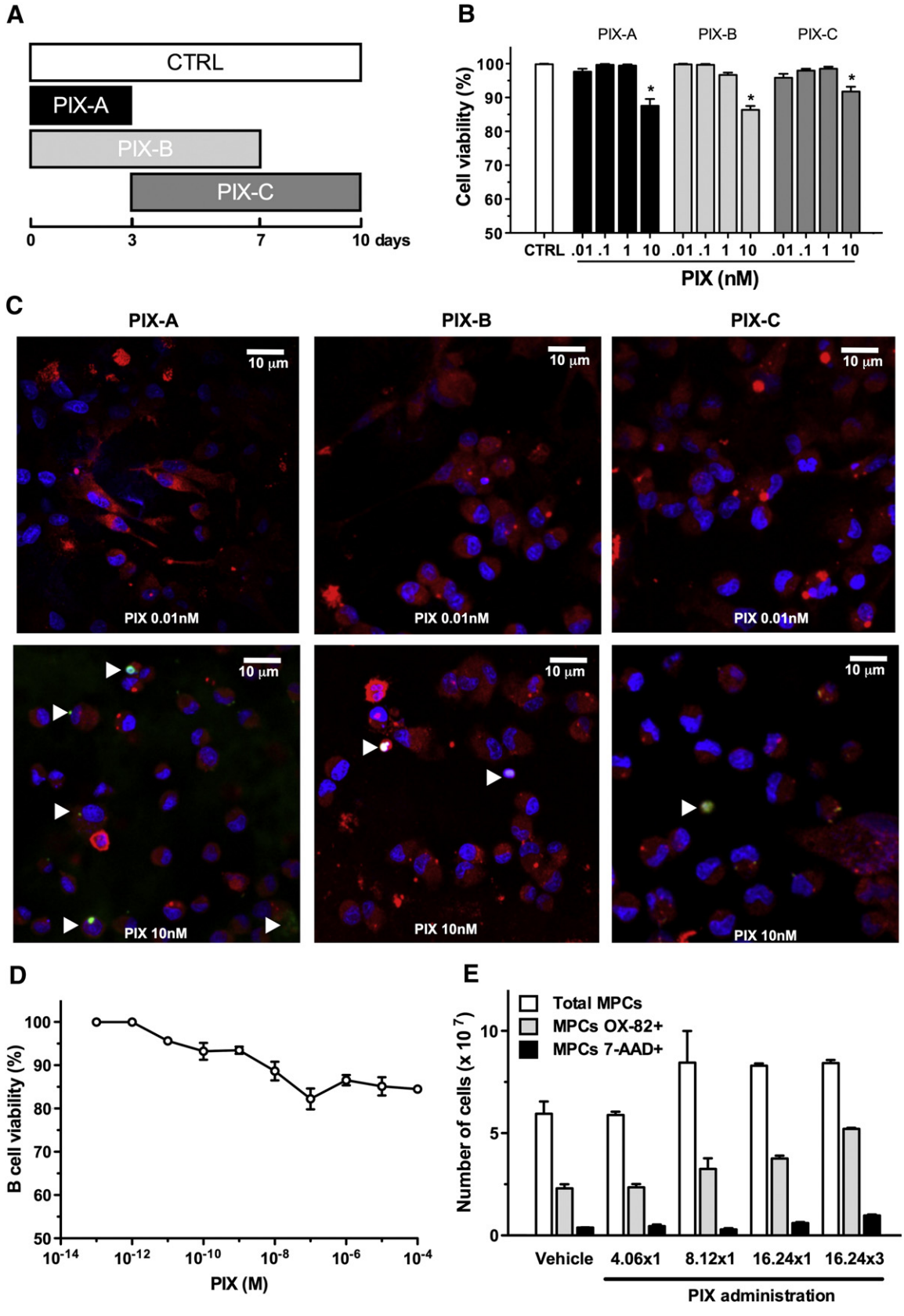
Therapeutic options for MG include corticosteroids and immunosuppressive drugs, pharmacological agents exerting a nonspecific generalized immune dysfunction (Conti-Fine et al., 2006). Although these treatments are effective in a large proportion of patients, in some cases the clinical response is unsatisfactory, or the severity of side effects may represent a limiting factor to their prolonged administration (Kumar and Kaminski, 2011). In this regard, new potent but well-tolerated immunosuppressive drugs are needed for treatment-resistant MG (Mantegazza et al., 2011; Cavalcante et al., 2012). MTX, a synthetic anthracenedione antineoplastic compound, has been approved by FDA for the treatment of progressive multiple sclerosis (Gonsette, 2007; Conway and Cohen, 2010). However, the prolonged use of this immunosuppressive drug is limited by the appearance of severe adverse effects, and new MTX-derived compounds have been developed. PIX was synthesized to reduce anthracycline-related cardiotoxicity without compromising antitumor efficacy, and it has been evaluated in the rat EAE model (Cavaletti et al., 2004; Mazzanti et al., 2005), showing amelioration of the disease manifestations, and long-lasting effect on lymphocyte subpopulations and APC. The observed effects were dose-dependent, and no signs of cardiotoxicity were observed. We reported the efficacy of the same total dose of PIX (16.24 mg/kg q7dx3, 1/4 of the $LD_{10}$, with preventive and therapeutic protocols) in EAMG, the animal model of MG, showing improvement of EAMG conditions and modulation of disease-related immunological parameters, without cardiotoxicity (Ubiali et al., 2008).

In order to better characterize PIX immunosuppressive properties, we investigated whether a reduced drug dosage in combination with modified schedule of treatments (thus lowering the drug cumulative load) were still able to ameliorate EAMG. We initially assessed whether an alternate-week treatment (PIX 16.24 mg/kg, q14dx3, T1 protocol) and a half-dosage weekly treatment (PIX 8.12 mg/kg, q7dx6, T2 protocol) could be as efficient as the previously published weekly treatment (PIX 16.24 mg/kg, q7dx3 (Ubiali et al., 2008)); our new results showed that the improved protocols ameliorated EAMG clinical symptoms, and these were associated with reduction of pathogenic anti self AChR antibodies and AChR-specific T helper proliferation. We also observed an even improved effect of the T2 protocol on EAMG, due to a more favorable PIX pharmacokinetic profile, allowing the achievement of a systemic steady-state level of the drug, estimated to be greater than 0.2 nM (Fig. 2).

A further lowered PIX dosage, with two different administration schedules (PIX 8.12 mg/kg, q14dx3, T3 protocol and PIX 4.06 × 6 mg/kg, q7dx6, T4 protocol), was also evaluated; however, despite the demonstration of a significant inhibition of AChR-specific T helper proliferation, reduction of the number of LNCs and modification of selected cytokine expression levels, neither protocols efficiently modulated EAMG symptoms. PIX bioavailability assay indicated a 60 pM drug level in the serum 24 h after administration (PIX 4.06 mg/kg), a level comparable with the IC50 for TAChR (25 pM) and R97–116 (63 pM) LNC responses from antigen-primed animals; instead, at 7 days the PIX serum levels dropped to 10 pM, below the IC50 values. Interestingly, PIX bioavailability in serum, after its administration at 8.12 mg/kg, showed a relatively stable level (300 pM and 200 pM, after 24 h and 7 days, respectively), and this may account for the effect of PIX protocol T2 but not T4. PIX levels in the serum of animals treated with PIX T3 and T4 protocols, are able to suppress LNC

proliferations, and were not cytotoxic for OX33$^{+}$ B cells, as demonstrated by the modest reduction (less than 5–8%) in cell viability.

Notably, our data highlighted that even one single low dose of PIX was sufficient to reduce the number of viable LNCs and to block immunocompetent cell proliferation; indeed, the amount of PIX in serum within 24 h from administration led to an efficient T cell suppression. It should be noted that we observed PIX mediated-suppression only in an activated immune system: PIX treatment (16.24 mg/kg × 1) in



naïve rats (HD/PIX) did not induce any alteration in the lymphoid cell compartment, differently from what we observed in TAChR-immunized animals; this may suggest that PIX does not interfere with the population of naïve resting T cells, present in not inflamed LNs, but does interfere with proliferating (antigen-activated) $CD4^+$ effector T cells.

The calculated PIX IC50 values seem to be specific with regard to the different cell subpopulations analyzed, with the lowest IC50 for homogeneous cultures of antigen-specific T cell lines, and with increasing values if heterogeneous cell populations, such as LNCs and SPNCs isolated from AChR-primed rats, were studied. SPNCs from not immunized (naïve) rats, and PBMCs from healthy human donors, were the most resistant to PIX cytotoxicity. This observation might be of particular relevance, in light of the possible use of PIX for the treatment of autoimmune disorders, as the therapy could be tapered to the appropriate low PIX dosage able to block only actively proliferating T cells. This in turn could be even more relevant if evaluated in the context of combination therapies, based on an initial short course of cytotoxic drugs able to induce antigen specific immunosuppression, followed by a conventional immunomodulation regimen with first-line drugs (induction–maintenance approach) (Conway and Cohen, 2010).

Our study has also demonstrated the feasibility of assessing in vitro the bioavailability of PIX in the serum; we are confident that this bioassay could be rapidly translated in the clinic, being a useful biomarker to carefully monitor the drug level in patients during PIX treatment.

Furthermore, we showed that the low PIX doses, cytotoxic to effector T cells, did not induce B cell death, did not show myelotoxic effects in vivo and did not alter the differentiation of bone marrow MPCs in iDCs in vitro; hence low dose-PIX treatment has a specific effect on actively proliferating effector T cells, without inducing cytotoxicity on other immune cell compartments. Our treatments showed a good pharmacokinetic profile and were able to ameliorate ongoing EAMG, with an administration schedule of a weekly single dose.

The response of MG to immunosuppression can be variable from patient to patient, and prolonged unresponsiveness can be frequently observed. Treatment-resistant myasthenia can be a real challenge for the clinician, particularly in patients with bulbar impairment. In this regard, the availability of new drugs is awaited.

Recently discovered drugs, such as the reversible inhibitor of inosine monophosphate dehydrogenase inhibitor mycophenolate mofetil (MMF), and the proteasome inhibitor Bortezomib (BZ) – both FDA approved for allograft rejection and cancer treatments – have been investigated in EAMG. Indeed, MMF proved to be an effective immunosuppressive drug in EAMG, blocking proliferation of T and B cells, but affecting also DC maturation and antigen presentation (Janssen et al., 2008). MMF is also currently prescribed to MG patients either alone or in association with prednisone (Hehir et al., 2010). Differently from MMF, BZ affects proteasome complex, thus blocking the NF-kB pathway and inducing apoptosis. BZ is, to date, the only drug able to efficiently inhibit terminally differentiated active plasma cells, thus being efficient in plasma cell mediated diseases such as mantle cell lymphoma and multiple myeloma, and therefore these preliminary results may suggest the rationale for their potential investigation in MG (Gomez et al., 2012). However, side effects, such as peripheral neuropathy, might be an issue in case of long-term treatment with BZ. In this respect, PIX may be of interest, either alone or in combination with other immunosuppressant to target both T and B cell counterparts of the disease. Therefore, our data provide the rationale for the proposal of clinical studies on PIX in MG to assess primarily its tolerability, and then its clinical efficacy as immunosuppressive compound in a T cell driven-B cell mediated disease.

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The authors declare that no conflict of interest exists.

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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.2013.02.021.

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**Fig. 4.** PIX effect on iDC differentiation, B cell and MPC viability. (A) Schematic representation of PIX treatment (10 pM–10 nM) during the differentiation of MPCs into iDCs (10 days): PIX-A: treatment day 0–day 3 (black bar); PIX-B: treatment day 0–day 7 (light gray bar); PIX-C: treatment day 3–day 10 (dark gray bar); CTRL: control treatment (white bar). (B) Cell viability of MPCs after PIX treatment, measured through fluorescence imaging and evaluated as the ratio between living and total $OX82^+$ labeled cells, excluding Tunel positive apoptotic cells, and compared to CTRL. Data from 3 samples/treatment condition, at least 5 image fields acquired per sample. p values: $^*p < 0.05$; (C) representative fluorescence images of data analyzed in (B): $OX82^+$ myeloid cells (red); Tunel positive cells (green); DAPI nuclei (blue). Magnification bar: 10 μm. White arrow-heads indicate Tunel positive dead cells. Upper row: PIX treatment 0.01 nM; lower row: PIX treatment 10 nM. (D). α-CD45RA positively selected B lymphocytes from LNs and SPN of healthy naïve rats and treated ex vivo for 3 days with increasing PIX concentration (10 pM–1 mM). Cell viability was evaluated via cytofluorimetry using 7-AAD as marker for dead cells and expressed as the ratio between living and total cell counts (cell viability, %) ± SE of triplicate measurements from two independent experiments. (E) Cytofluorimetric quantification of BM derived MPC viability in rats treated i.v. with a single dose of PIX ($4.06 \times 1$, $8.12 \times 1$, $16.24 \times 1$), three weekly doses of PIX ($16.24 \times 3$) or vehicle (saline solution) and sacrificed after 7 days after the last PIX injection. Data are representative (mean cells number ± SE) of three independent experiments. Total MPCs (white bars), $OX82^+$ myeloid cells (gray bars) and $7\text{-}AAD^+$ dead cells (black bars) are analyzed via cytofluorimetry.

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