Paractin interferes with T cell activation and reduces Experimental Autoimmune Encephalomyelitis in the Mouse*.

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Abbreviations used in this paper: DC, dendritic dells; OVA, Ovalbumin; MS, multiple sclerosis; EAE, Experimental Autoimmune Encephalomyelitis; DTH, delayed type hypersensitivity; NP-BSA, 4-hydroxy-3-nitrophenyl-acetyl conjugated to Bovine Serum Albumin; NP-CGG, 4-hydroxy-3-nitrophenyl-acetyl conjugated to Chicken Gamma globulin Recommended section assignment: Inflammation and Immunopharmacology

ABSTRACT

Paractin is a proprietary blend of Andrographolide, 14-DeoxyAndrographolides, and NeoAndrographolides, a bicyclic diterpenoid lactone derived from extracts of *Andrographis paniculata*, a plant indigenous to South Asian countries that shows anti-inflammatory properties. The molecular and cellular bases for this immunomodulatory capacity remain unknown. Here we show that Paractin is able to downmodulate both humoral and cellular adaptive immune responses. *In vitro,* this molecule was able to interfere with T cell

proliferation and cytokine release in response to allogenic stimulation. These results were consistent with the observation that T cell activation by dendritic cells (DCs) was completely abolished by exposing DCs to Paractin during antigen pulse. This molecule was able to interfere with maturation of DCs and with their ability to present antigens to T cells. Furthermore, *in vivo* immune responses such as antibody response to a thymus-dependent antigen and delayed type hypersensitivity were drastically diminished in mice by Paractin treatment. Finally, the ability of Paractin to inhibit T cell activation was applied to interfere with the onset of Experimental Autoimmune Encephalitis, an inflammatory demyelinating disease of the central nervous system that is primarily mediated by CD4+ T cells and serves as an animal model for human Multiple Sclerosis. Treatment with Paractin was able to significantly reduce EAE symptoms in mice by inhibiting T cell and antibody responses directed to myelin antigens. Our data suggest that Paractin is able to efficiently interfere *in vitro* and *in vivo* with T cell activation, a feature that could be useful for interfering with detrimental T cell responses.

INTRODUCTION

Multiple Sclerosis (MS) is a chronic neuroinflammatory demyelinating disorder of the central nervous system (CNS) that predominantly affects young adults. There are approximately 1.1 million cases worldwide (Noseworthy et al., 2000). Although the aetiology of the progressive neurological loss has not yet been fully elucidated, it is believed that environmental stimuli and genetically determined susceptibility are both implicated in the detrimental autoimmune response against components of the myelin sheath (Lucchinetti et al., 2001; Visconti et al., 2003). Empirical evidence points toward an autoimmune pathogenesis, where myelin-specific CD4+ and CD8+ T cells are thought to play a central role by reacting against and destroying the myelin sheath (Wingerchuk et al., 2001). Plaques of demyelination with perivascular inflammation, axonal damage and oligodendroglia destruction are scattered throughout the white matter of the CNS. Autoimmune destruction of myelin leads to a long-term breakdown of saltatory conduction in myelinated fibers with subsequent impairment of neuronal function and axonal loss (Lucchinetti et al., 2000; Lassmann et al., 2001; Hemmer et al., 2002;

Lassmann, 2002). The disease results in multiple neurologic symptoms and signs, which are often disabling and show exacerbations and remissions that, can lead to a chronic progressive course in later years (Bruck et al., 2002).

Even if our understanding of the mechanisms that underlie MS has progressed significantly throughout the last years, our means for therapeutic intervention are still very limited. Current therapies available include the use of β-interferon, glatiramer acetate, corticosteroids and cyclophosphamide; however, none of these immunosuppressive or experimental treatments offers a curative option (Noseworthy et al., 2000; Polman and Uitdehaag, 2003; Visconti et al., 2003). Thus, MS treatment remains a major research challenge that needs significant efforts for the development of novel and efficient therapeutic approaches.

Due to the fact that it shows close similarity to clinical and histopathological aspects of human MS, experimental autoimmune encephalomyelitis (EAE) represents a suitable animal model for testing efficacy of potential therapeutic agents for MS (Sun et al., 2001; Kuchroo et al., 2002). In C57BL/6 mice, EAE can be induced by injection of a peptide derived from mouse Myelin Oligodendrocyte Glycoprotein (MOG), which leads to chronic spinal cord demyelination and paralysis. EAE is characterized by focal areas of inflammation and demyelination throughout the CNS, with axonal loss that results in ascending paralysis involving first the tail and then the limbs (Iglesias et al., 2001).

Favorable immune modulation observed in response to plant-derived molecules for several inflammatory diseases, such as arthritis and MS (Killestein et al., 2003; Shin et al., 2003; Soeken et al., 2003), provides encouragement for the potential use of those preparations to treat autoimmune disorders. In this direction, extracts from *Andrographis paniculata*, a plant indigenous to Southeast Asian countries, have been shown to modulate inflammatory immune responses (Wang et al., 2004). One of the molecules present in the extracts of *A. paniculata*, a bicyclic diterpenoid lactone known as andrographolide, has been reported in several assays to be particularly efficient at regulating the immune response (Calabrese et al., 2000; Rajagopal et al., 2003). This molecule has recently been shown to work as an anti-inflammatory agent by reducing the generation of reactive oxygen species in human neutrophils (Shen et al., 2002), as

well as preventing microglia activation (Wang et al., 2004).

However, the molecular and cellular mechanisms responsible for the immunomodulatory properties of Andrographis remain unknown, as well as the potential *in vivo* anti-inflammatory effects resulting from treatment with this extract. Here we provide evidence for a role of Paractin Andrographis extract as an inhibitor for T cell-mediated immune responses *in vitro* and *in vivo*. *In vitro,* we show that T cell activation by antigen-pulsed DCs can be completely abolished by exposing DCs to Paractin during antigen pulse. In addition, Paractin interfered with T cell proliferation and cytokine release in response to allogenic stimulation. *In vivo*, Paractin drastically diminished antibody response to a T-dependent antigen, as well as a delayed type hypersensitivity response. Consistent with these findings, mice treated with Paractin showed a significant reduction of EAE symptoms, as well as reduced T cell and antibody responses to myelin antigens, compared to control mice. These data indicate that Paractin can interfere with T cell activation, and support the notion that the immunosuppressive properties of this molecule could be applied for treating autoimmune diseases.

MATERIALS AND METHODS

Animals.

Six to eight weeks old female C57BL/6 mice were used in these experiments and kept under pathogen-free conditions at the animal core facility of the Pontificia Universidad Católica de Chile. All animal work was performed according to institutional guidelines.

Reagents and synthetic peptides.

Paractin and Pure Andrographolide was kindly provided by HP Ingredients. A stock solution for this molecule was prepared by dissolving andrographolide in dimethylsulphoxide (DMSO) at 50mM, which was then serially diluted in PBS immediately prior to experiments. Myelin oligodendrocyte glycoprotein-derived peptide (MOG35-55, MEUVGYRSPFSRUVUVHLYRNGK), Chicken Egg Ovalbumin (OVA) peptide SIINFKEL

(OVA257-265, for presentation on H- 2Kb) and OVA peptide TEWTSSNVMEERKIKV (OVA265-280, for presentation on I-Ab) were synthesized by solid-phase method using Fmoc chemistry on an automated 433A peptide synthesizer (Applied Biosystems, Foster City, CA) at the Peptide Synthesis Facility of the Albert Einstein College of Medicine. All peptides were purified to >98% homogeneity by reversed-phase HPLC on a Vydac C-18 column (2.1 or 4.6 mm x 25 cm, 300 Å) using HP-1090M HPLC (Hewlett Packard). The identity of the purified peptide was determined by a tandem quadrupole mass spectrometer (TSQ700, Finnigan MAT, San Jose, CA).

EAE induction

Six to eight weeks old female C57BL/6 mice were injected subcutaneously with 50 ug of MOG35-55 peptide emulsified in Complete Freund's Adjuvant (Gibco, BRL) supplemented with heat-inactivated *Mycobacterium tuberculosis* H37Ra (Difco Laboratories). In addition, mice received intraperitoneal injections with pertussis toxin (500 ng, Calbiochem) at the time of sensitization and 48 hours later. Clinical signs of disease were seen usually between day 15 and 18 after sensitization and assessed daily according to the following scoring criteria: 0 = no detectable signs of EAE; 1 = flaccid tail; 2 = hind-limb weakness or abnormal gait; 3 = complete hind-limb paralysis; 4 = paralysis of fore and hind limbs; 5 = moribund or death. In order to prevent unnecessary animal suffering, mice severely affected by the disease were euthanized with the supervision of a veterinarian. Data shown are means of daily measured clinical score that were obtained from 4 independent experiments.

Paractin treatment

Mice were treated intraperitoneally with a daily dose equal to 13 mg/kg of Paractin in PBS (total volume of 100 µl). Treatment started one week before MOG sensitization and continued through all the experiment. As controls, age matched female mice were sensitized with MOG, but not treated with Paractin. Treated and control mice were clinically evaluated on a daily basis. At the doses used, Paractin was well tolerated by mice and no evidence of toxicity was observed.

DCs, antigen-presentation assay and T cell hybridoma activation

Bone marrow-derived DCs were prepared as previously described (Inaba et al., 1992; Lopez et al., 2000; Kalergis and Ravetch, 2002). Briefly, DCs were grown from bone marrow progenitors in RPMI 1640 containing 5% FCS supplemented with GMCSF (50 U/ml). Day 5 DCs were treated with andrographolide 10 μM for 24 hrs. After this time, DCs were pulsed for 16 hours either with OVA protein or OVA peptide (OVA257-265 for presentation on H-2Kb or OVA265-280 for presentation on I-Ab). After the pulse, DCs were washed and co-cultured at different ratios with either 1x105 B3Z or 1x105 OT4H T-cell hybridomas. B3Z and OT4H are specific for H-2Kb/OVA257-265 and I-Ab/OVA265-280, respectively and secrete IL-2 upon TCR stimulation (Shastri and Gonzalez, 1993). After 20 hours of DC-T cell co-culture, IL-2 from supernatants was measured by cytokine ELISA as previously described (Kalergis et al., 2000; Kalergis and Nathenson, 2000; Kalergis et al., 2001). DC viability was determined by Trypan blue exclusion.

T cell cytokine release assays

Inguinal and mesenteric lymph nodes were obtained on day 21 after EAE induction from control or Paractin treated mice. Cellular suspensions from these lymph nodes were cultured (5x105 cells/well) in RPMI 1640 containing 5% FCS with different concentrations of MOG35-55 peptide. Cultures were incubated in 96-well round bottom plates for 48 hrs at 37ºC in a cell culture incubator. IL-2 release in response to MOG35-55 peptide was determined on culture supernatants by cytokine ELISA as previously described (Kalergis et al., 2000; Kalergis and Nathenson, 2000; Kalergis et al., 2001). IFN-γ release was also determined by cytokine ELISA, but using purified anti-mouse IFN-γ (clone R4-6A2, PharMingen,) as capture antibody and biotin rat antimouse IFN-γ (clone XMG1.2 PharMingen) as detection antibody.

Measurement of Anti-MOG antibody response

Mouse sera obtained on days 7 and 21 after sensitization with MOG35-55 peptide were analyzed for the presence of MOG-specific IgG by ELISA. Briefly, ELISA plates (Falcon) were coated at 4ºC overnight with 10 μg/ml MOG35-55 peptide in 0.1 M NaHCO3 buffer (pH 8.4) and then blocked with PBS-BSA 1% for 2h at RT. Serum samples were diluted in PBS-BSA 1% starting at 1:60 and incubated for 3h at room temperature. IgG was detected with rabbit anti-mouse IgG antibody conjugated to horse-radish peroxidase

(Amersham_Pharmacia). After extensive washing, HRP substrate was added (3,3',5,5' tetramethylbenzidine, Sigma) and plates were read at OD450nm on a micro plate reader.

DC maturation assays

DCs were treated with andrographolide 10 µM for 24 h and then incubated with LPS (1 ug/ml) for 36 h. Untreated control DCs were included in all the experiments. After LPS treatment, cells were analyzed for expression of surface markers I-Ab, CD86 and CD40 on a FACScan flow cytometer (Becton Dickinson). To evaluate DC maturation, DCs were double-stained with anti-CD11c-PE (clone HL3, PharMingen) plus either anti-I-Ab-FITC (clone AF6-120.1, PharMingen), anti-CD86-FITC (clone GL1, PharMingen), or anti-CD40-FITC (clone 3/23, PharMingen), fixed on PFA (1%, PBS) and analyzed by FACS. To determinate the densities of H-2Kb/OVA complexes on the surface of DCs, OVA-pulsed cells were stained with anti-CD11c-PE and 150 µl of 25-D1.16 supernatant (mouse kappa-IgG1 mAb specific for the H-2Kb/SIINFEKL complex). After washing, goat anti-mouse IgG-FITC (PharMingen) was added to the DCs. Cells were washed in PBS, fixed on PFA (1%, PBS) and analyzed by FACS.

Mixed lymphocyte reaction

Lymph node cell suspensions obtained from C57BL/6 and Balb/c mice were cocultured in 96-well round bottom plates at 1x105 cells per strain on each well for 72 h. After this time, supernatants were harvested and analyzed for IL-2 release by cytokine ELISA as described above. T cell proliferation was assessed using CellTiter Cell Proliferation Assay (Promega) following the methodology provided by the manufacturer.

NP-specific antibody response.

Mice were immunized subcutaneously with 50 µg 4-hydroxy-3- nitrophenylacetyl conjugated to bovine serum albumin (NP17-BSA, Biosearch Technologies, Inc.) in Alum (Pierce). Seven and 14 days after immunization, Npspecific IgG antibodies were measured in mouse sera by ELISA. Briefly, plates were coated at 4ºC overnight with NP23-CGG (0.5 ug/well) in 0.1 M NaHCO3 buffer (pH 9.5) and anti-NP IgG antibodies were detected as described above.

Delayed-type hypersensitivity reaction

Mice were immunized subcutaneously with 100 µg of OVA (Calbiochem) emulsified in complete Freund's adjuvant (Gibco, BRL). One week after immunization, animals were intracutaneously challenged in the ear with 50 µg OVA dissolved in 20 µl of PBS. Ear thickness was measured at different times after challenge with a micrometer (Mitutoyo, Japan). Increased ear thickness was expressed as the means of at least three measurements per mouse in millimeters x 10-2 ± SE.

RESULTS

In vitro T cell activation is inhibited by Paractin.

The ability of Paractin to interfere with T cell activation was evaluated on a mix lymphocyte reaction between C57BL/6 and Balb/C splenocytes. As shown in Figure 1A, in this assay, T cell proliferation and IL-2 release were inhibited by Paractin in a dose dependent fashion. No measurable effect on background proliferation and IL-2 release was observed.

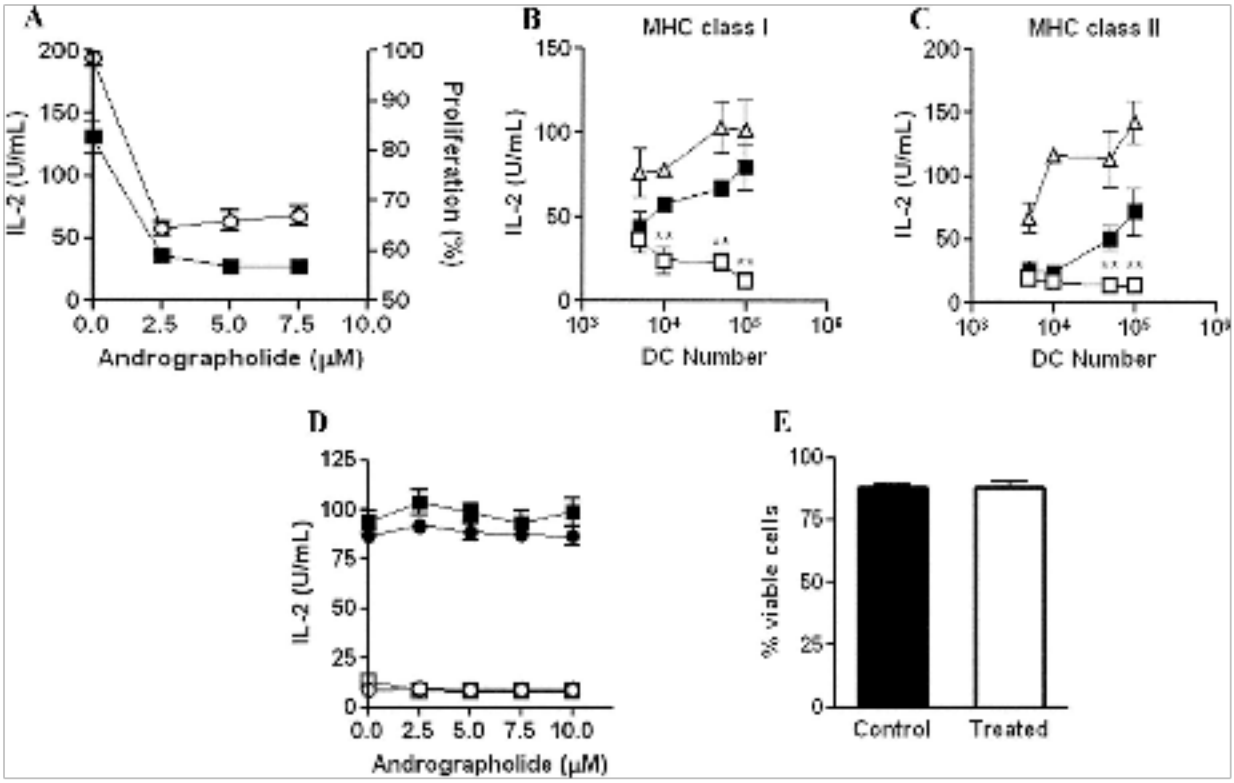

Fig. 1. In vitro T cell activation is inhibited by Paractin. A, mixed lymphocyte reaction is inhibited by Paractin. Lymph node cell suspensions from C57BL/6 and BALB/c mice were coincubated as described under *Materials and Methods*. Paractin was added at the indicated concentrations to the cells. Proliferation (filled squares) and IL-2 release (empty circles) were determined after 72 h of culture. B and C, T cell activation by antigen-pulsed DCs is suppressed by Paractin. Control DCs (filled squares) and Paractin-treated DCs (empty squares) were pulsed with OVA protein and cocultured either with H-2K^b/OVA$_{257-264}$-specific (B) or I-A^b/OVA$_{265-280}$-specific (C) T cell

hybridomas. As controls, Paractin-treated DCs were pulsed with OVA-peptides SIINFEKL or TEWTSSNVMEERKIKV for presentation on H-2K^b or I-A^b, respectively (empty triangles) (**, $p < 0.01$ when compared with controls, Student's *t* test). D, Paractin treatment did not affect antigen-independent T cell activation. B3Z (filled squares) or OT4H (filled circles) T cell hybridomas were stimulated with plate-bound anti-CD3ϵ. Plates treated with vehicle were included as controls (empty squares for B3Z and empty circles for OT4H). Paractin was added at the indicated concentrations, and IL-2 release was determined after 24 h of culture. E, DC viability remains unaffected after treatment with Paractin, based on trypan blue exclusion assay. Data shown are means of at least four independent experiments.

To determine whether this was an effect on the T cells or the APCs, an antigen presentation assay was set up with bone marrow-derived DCs pulsed *in vitro* with OVA and co-cultured either with H-2Kb/OVA257-265- or I-Ab/OVA265-280-specific T cell hybridomas (B3Z and OT4H, respectively). As shown in Figure 1B and 1C, treating DCs with Paractin before OVA-pulse prevented them from activating both CD4+ and CD8+ OVA-specific T cell hybridomas. This inhibition was only observed when Paractin-treated DCs were pulsed with whole OVA protein, and not when OVA257-265- or OVA265-280 peptides were exogenously added to these cells (Figure 1B, 1C). Consistent with these findings, Paractin treatment had no effect on APC-independent T cell activation with anti-CD3 (Fig. 1D). In addition, trypan blue exclusion assays show that viability of DCs remains unaffected after treatment with 30 µM Paractin (Figure 1E). These data are supported by measurements of mitochondrial function showing that concentrations up to 150 µM Paractin did not affect cell viability.

Thus, our results suggest that, at the concentration tested, Paractin inhibits the ability of DCs to process OVA and generate the peptide-MHC complexes required for T cell activation. To test this notion, the ability of Paractin-treated DCs to process and present OVA derived peptides on MHC-I was evaluated using an H-2K^b/OVA$_{257-264}$-specific monoclonal antibody (Porgador et al., 1997). In these assays, Paractin was able to prevent processing and presentation of OVA peptides on the MHC-I molecule H-2K^b (Figure 2A).

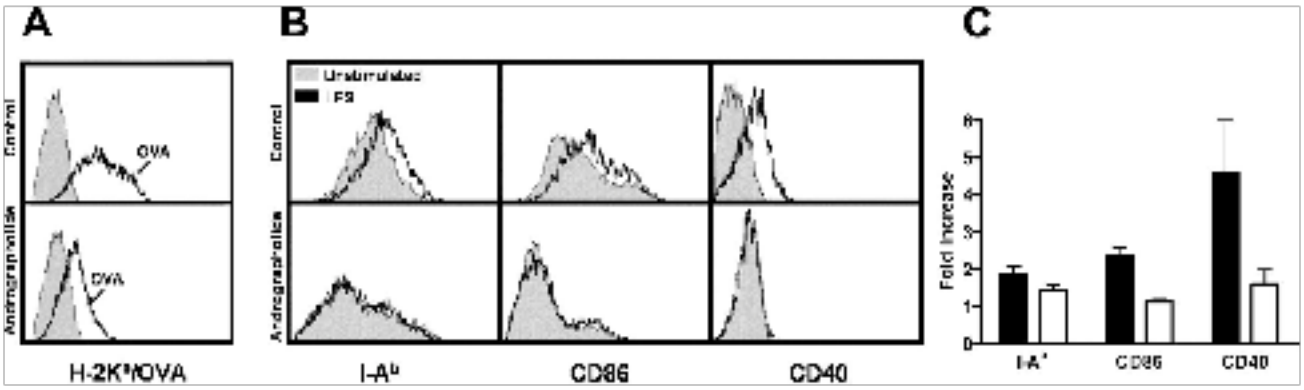

Fig. 2. Antigen processing by DCs and DC maturation are inhibited by Paractin. A, ability of DCs to process and present OVA-derived peptides on H-2K^b was abolished by Paractin (top, untreated DCs; bottom, treated DCs). Histograms show fluorescence intensity from binding of 25-D1.16 mAb to H-2K^b/SIINFEKL on CD11c$^+$ cells (shaded and clear histograms show control or OVA-pulsed DCs, respectively). B, up-regulation of maturation markers I-A^b, CD40, and CD86 in response to LPS is inhibited by Paractin. Histograms show surface expression of maturation markers on CD11c$^+$ cells. Shaded histograms indicate immature DCs, and white histograms represent LPS-treated DCs (top, control DCs; bottom, treated DCs). C, bar graphs show the increase of mean fluorescence intensity for I-A^b, CD86, and CD40 (CD11c$^+$ gate). Black bars represent control DCs, and white bars indicate Paractin-treated DCs. Data shown are means of at least three independent experiments.

To further evaluate the effect that Paractin could have on DC function, maturation of DCs was induced by LPS treatment in the presence of Paractin. As shown in Fig. 2, B and C, Paractin also inhibited up-regulation of the maturation markers I-A^b, CD40, and CD86 (B7.2) in response to LPS.

In vivo T cell function is suppressed by Paractin treatment.

The data shown above suggested that Paractin could be able to interfere with the initiation of an immune response, by inhibiting T cell activation by antigen loaded DCs. To test whether Paractin was also able to affect *in vivo* immune responses, C57BL/6 mice were treated with Paractin and immunized with NP_{17}-BSA (a thymus-dependent antigen) adsorbed to alum. Seven days post-immunization, anti-NP IgG titers were determined by ELISA using NP23-CGG as antigen. Compared to untreated controls, significantly reduced anti-NP IgG titers were observed for Paractin treated mice (Figure 3A).

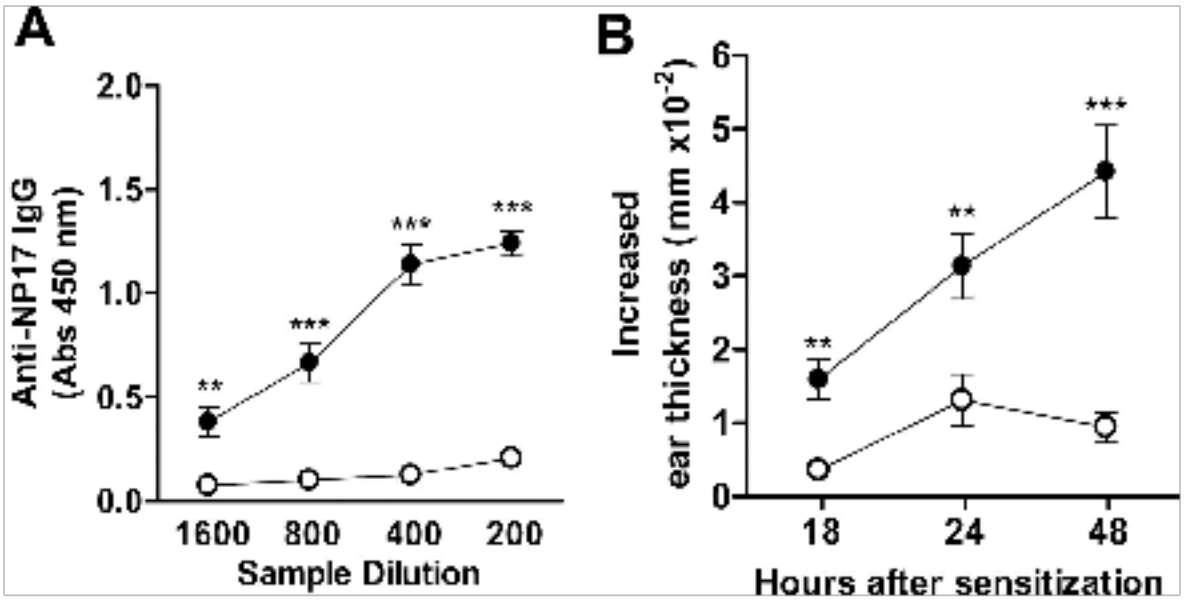

Fig. 3. In vivo T cell function is suppressed by Paractin treatment. A, T cell-dependent antibody production is diminished by Paractin. Control (filled circles) and Paractin-treated C57BL/6 (empty circles) were immunized with NP_{17}-BSA as described under *Materials and Methods*. Seven days postimmunization, anti-NP IgG titers were determined by ELISA using NP_{25}-CGG as antigen (**, $p < 0.01$; ***, $p < 0.001$, Student's *t* test). B, delayed-type hypersensitivity is reduced by Paractin treatment. Control (filled circles) and Paractin-treated C57BL/6 (empty circles) mice were immunized with OVA as described under *Materials and Methods*. One week later, mice were challenged on the ear with OVA dissolved in PBS. Ear thickness was measured at different times after challenge (**, $p < 0.01$; ***, $p < 0.001$, Student's *t* test). Data shown are means of two independent experiments.

To evaluate the effect of Paractin directly on T cell effector function, delayed type hypersensitivity (DTH) against OVA was induced in control and Paractin treated mice. As shown in Figure 3B, Paractin almost completely suppressed DTH induced by OVA immunization and challenge in C57BL/6 mice.

Paractin treatment significantly reduces severity of Experimental Autoimmune Encephalomyelitis in the mouse.

To evaluate whether inhibition of T cell activation and antibody production by Paractin could modulate an autoimmune response, C57BL/6 mice were treated with Paractin and induced to develop EAE by injection of MOG. As shown in Figure 4, control (solvent injected) C57BL/6 mice presented signs of disease on day 14 after sensitization. The progression of EAE symptoms was equivalent with the observed in previous reports (Bright et al., 2003; Gilgun-Sherki et al., 2003). In contrast, treatment with Paractin not only delayed the onset of EAE, but also significantly reduced the severity and incidence of disease (Figure 4 and Table 1).

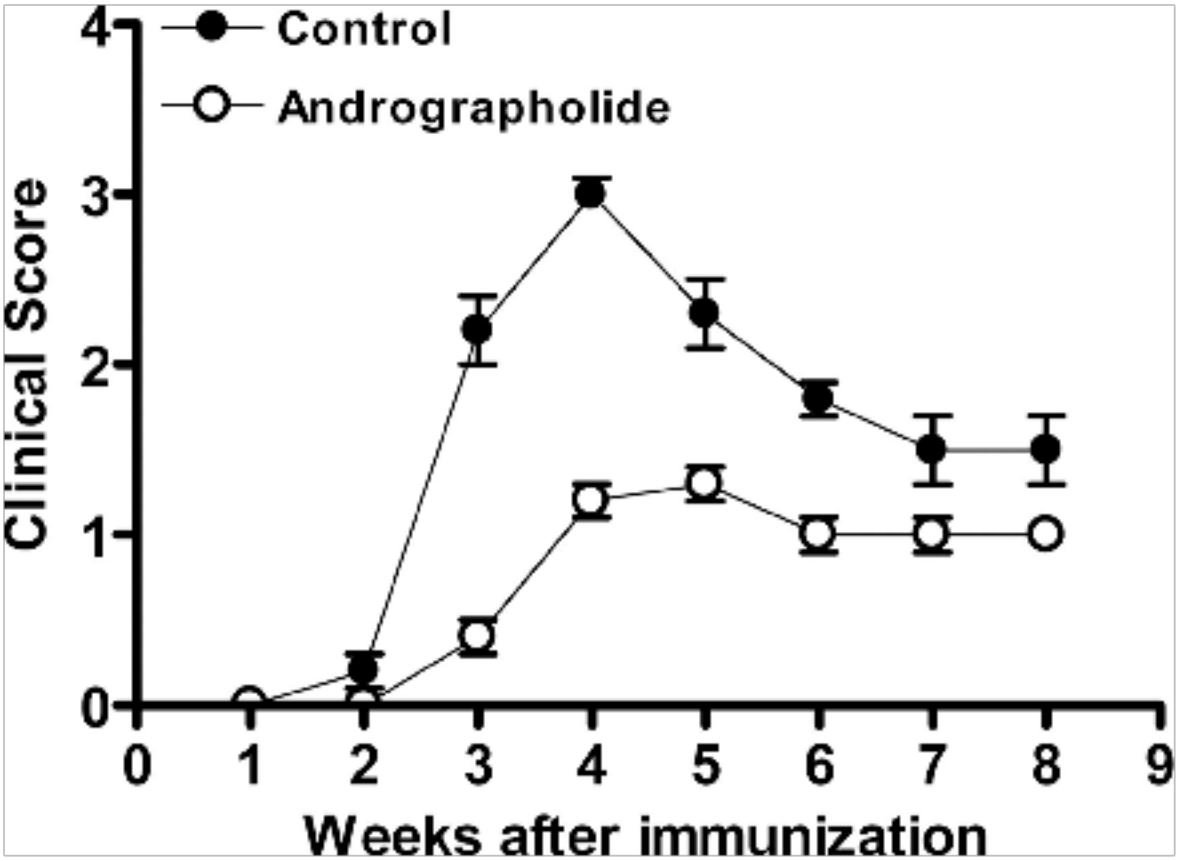

Fig. 4. Experimental autoimmune encephalomyelitis is reduced by Paractin treatment. EAE was induced by immunizing control (filled circles) or Paractin-treated (empty circles) mice with MOG_{35-55} peptide as described under *Materials and Methods*. Clinical score was determined according to the criteria described under *Materials and Methods* ($p < 0.05$, from Mann-Whitney rank sums two-tailed analysis, applied to the entire data set). The analyses for each time period after immunization between the two groups show statistically significant differences during peak severity (weeks 3-6, $p < 0.001$, Student's *t* test) and no significant differences in residual clinical signs (after 6 weeks). Data shown represent average weekly clinical scores of all animals in the group and are means of four independent experiments.

TABLE 1 Summary of EAE disease parameters

EAE was induced by immunizing control or andrographolide-treated mice with MOG_{35-55} peptide, as described under *Materials and Methods*. Clinical score was evaluated on a daily basis, and mean maximum score was calculated by adding clinical scores at the peak of disease for animals that develop EAE in each group and then divided by the total number of animals.

Group	Incidence	Maximum Score	Mean Maximum Score	Day of Onset
			mean + S.E.	
Control	14/14 (100%)	5	3.7 ± 0.1	14.7 ± 0.2
Paractin	8/17 (47%)	3	1.9 ± 0.1*	17.6 ± 0.5*

* $p < 0.05$, compared with the control, unpaired Student's t test.

Reduced anti-myelin T cell and antibody response in Paractin treated mice.

The diminished EAE incidence and severity resulting from Paractin treatment in the mouse could be due either to an interference with autoreactive T cell activation and antibody production or to a nonspecific anti-inflammatory effect of this compound. To approach this issue, 3 weeks after EAE induction, lymph nodes were obtained from control and Paractin-treated mice to evaluate cytokine release in response to MOG peptide. As shown in Fig. 5A, IFN-γ and IL-2 secretion was observed only in lymph node suspensions obtained from untreated mice suffering from EAE. In contrast, neither IFN-γ nor IL-2 could be detected in supernatants from MOG-stimulated lymph node cell suspensions derived from Paractin-treated mice. Consistent with these observations, anti-MOG IgG could only be measured in sera from control animals suffering from EAE, whereas Paractin-treated animals showed an almost complete absence of anti-MOG antibody titers (Fig. 5B). Thus, it seems likely that Paractin treatment reduced EAE severity by impairing T cell priming by DCs, which could also indirectly affect antibody production against myelin antigens.

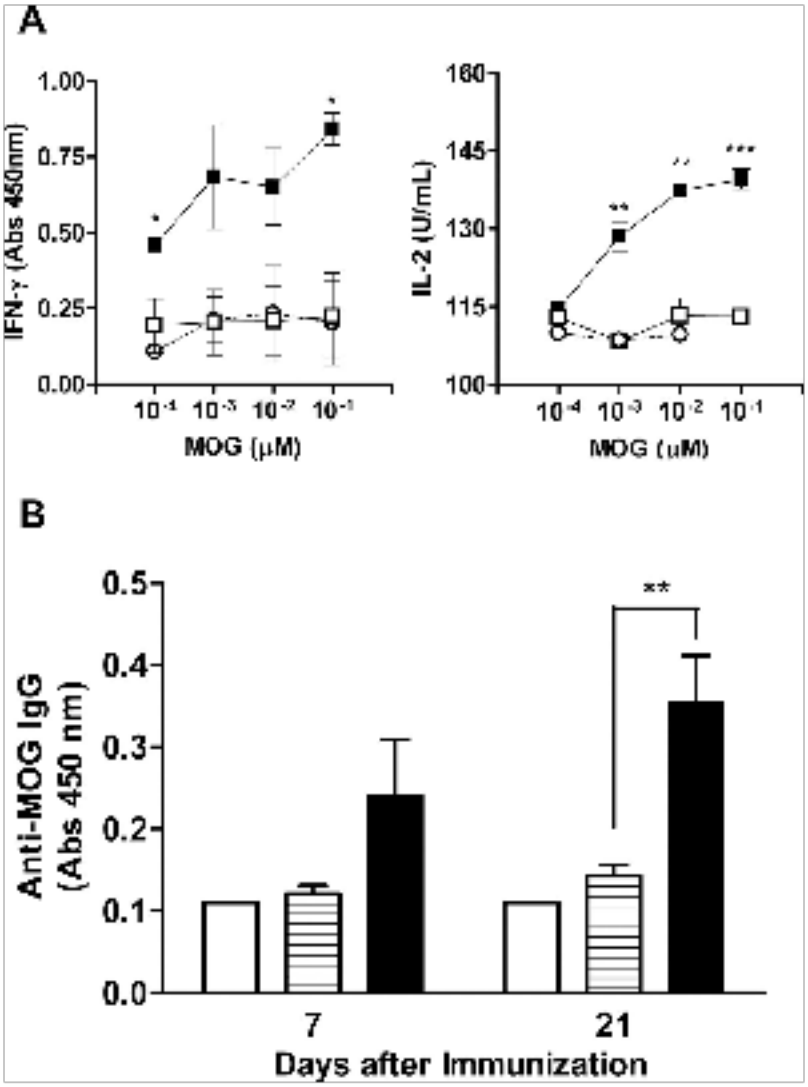

Fig. 5. Reduced antimyelin T cell and antibody response in andrographolide-treated mice. A, three weeks after EAE induction, lymph nodes were obtained from control (filled squares), andrographolide-treated (empty squares), and naive (empty circles) mice to evaluate cytokine release (IL-2 and IFN-γ) in response to MOG peptide (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Student's *t* test). B, sera were obtained from the same animals, and anti-MOG specific IgG titers were measured by ELISA. Control mice are shown as black bars, andrographolide-treated mice as shaded bars, and naive mice as white bars (**, $p < 0.01$, Student's *t* test). Data shown are means of three independent experiments.

DISCUSSION

New immunomodulatory therapeutic strategies are required to prevent or treat autoimmune diseases, such as multiple sclerosis. Here we provide evidence for a potential role of a blend of bicyclic diterpenoid lactone---andrographolide, 14-Deoxyandrographolides, and

Neoandrographolides as an inhibitor of T cell mediated immunity that ameliorates EAE in the mouse. When tested on a series of *in vitro* and *in vivo* assays, Paractin was shown to interfere with T cell activation and function.

Paractin was able to prevent OVA-pulsed DCs from activating either $CD4^+$ or $CD8^+$ T cell hybridomas. This result is consistent with the absence of H-$2K^b$/$OVA_{257-264}$complexes on the surface of DCs treated with Paractin at the time of OVA pulse. Thus, it seems likely that treatment with this extract prevented processing and presentation of OVA peptides on MHC molecules to OVA-specific T cells.

In addition to impair generation of peptide-MHC complexes, Paractin reduced the efficiency of DC maturation in response to LPS. Thus, when fold increase in surface expression was evaluated, reduced LPS-induced up-regulation of maturation markers I-A^b, CD40, and CD86 (B7.2) was observed as result of Paractin treatment. Although it was apparent that Paractin slightly modified basal expression of I-A^b, CD86, and CD40, these differences were not statistically significant. The ability to interfere with DC maturation suggests an explanation for the inhibition of T cell activation observed in vivo after Paractin treatment (see below).

Accordingly, the in vitro inhibition of T cell activation caused by Paractin is consistent with the suppression of the immune response in the mouse, as shown on three different experimental assays designed to measure immune system function in vivo. Thus, antibody (IgG) secretion against the T cell-dependent antigen NP_{17}-BSA was significantly reduced by Paractin treatment. Similarly, the DTH response against the antigen OVA was diminished to background levels by treatment with Paractin. These results support the notion that T cell-mediated immune responses can be effectively impaired by this molecule.

Finally, we evaluated whether the capacity of Paractin to impair T cell activation could be applied to prevent the onset of EAE in mice. As shown in Fig. 4 and Table 1, Paractin treatment significantly reduced both the incidence and clinical severity of EAE in C57BL/6 mice during early phase of disease. Residual clinical signs were not significantly changed by treatment with this molecule. Clinical data were consistent with the observation that lymph

node cellular suspensions derived from Paractin-treated mice showed reduced IFN-γ and IL-2 release in response to MOG (Fig. 5A), two important pro-inflammatory cytokines that participate in EAE pathogenesis (Lassmann et al., 2001; Lucchinetti et al., 2000; Wingerchuk et al., 2001). These data support the notion that the beneficial effects of Paractin are mediated preferentially by specific interference with antigen presentation by DCs and, thus, with T cell activation, and they correlate with the clinical scores shown by the animals. Further research is required to evaluate the potential therapeutical capacity of Paractin when administered after symptoms of EAE have started.

The reduced in vivo T cell priming, which is probably responsible for the decreased DTH and EAE responses observed in Paractin-treated mice, could result from the impairment on DC maturation and generation of peptide-MHC complexes caused by Paractin. However, whether this molecule is directly altering DC function in vivo remains to be evaluated.

In addition to activation of autoreactive T cells during the sensitization phase, EAE pathogenesis involves several inflammatory mediators, which are also responsible for myelin damage. Recent studies provide evidence suggesting that Androhraphis could also interfere with the function of inflammatory cells such as neutrophils and microglia (Batkhuu et al., 2002; Shen et al., 2002; Wang et al., 2004). Because these inflammatory cells have been implicated in the pathogenesis of inflammation in MS (Calabrese et al., 2002; Smith and Lassmann, 2002; Hill et al., 2004), the relative contribution of Paractin Andrographis extract to diminish adaptive versus nonspecific inflammation needs to be defined. Furthermore, an antiapoptotic activity has been shown for andrographis (Chen et al., 2004), which could also contribute to reduce severity of EAE in the mouse by increasing neuronal resistance to cell death induced by local inflammation. Thus, it is likely that Paractin Andrographis extract interferes with EAE by preventing activation of autoreactive T cells and by reducing inflammatory damage.

In summary, the data presented here suggest that Paractin is able to modulate T cell activation both *in vitro*, as well as *in vivo*. The exact mechanism by which Paractin exhibits its beneficial effect on EAE is still unknown, but we provide evidence it could prevent initial T cell priming

by interfering with DCs maturation process and antigen presentation properties. Therefore, Paractin may be a promising therapeutic agent for the treatment of autoimmune diseases, such as multiple sclerosis.

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FOOTNOTES

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