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Paradoxical Effect of Cortistatin Treatment and Its Deficiency on Experimental Autoimmune Encephalomyelitis

Luciana Souza-Moreira,* Maria Morell,* Virginia Delgado-Maroto,* Marta Pedreño,* Laura Martinez-Escudero,*† Marta Caro,* Francisco O’Valle,‡ Raul Luque,* Milagros Gallo,‡ Luis de Lecea,‡* Justo P. Castaño,§ and Elena Gonzalez-Rey*||

Cortistatin is a cyclic-neuropeptide produced by brain cortex and immune cells that shows potent anti-inflammatory activity. In this article, we investigated the effect of cortistatin in two models of experimental autoimmune encephalomyelitis (EAE) that mirror chronic and relapsing-remitting multiple sclerosis. A short-term systemic treatment with cortistatin reduced clinical severity and incidence of EAE, the appearance of inflammatory infiltrates in spinal cord, and the subsequent demyelination and axonal damage. This effect was associated with a reduction of the two deleterious components of the disease, namely, the autoimmune and inflammatory response. Cortistatin decreased the presence/activation of encephalitogenic Th1 and Th17 cells in periphery and nervous system, and downregulated various inflammatory mediators, whereas it increased the number of regulatory T cells with suppressive effects on the encephalitogenic response. Moreover, cortistatin regulated glial activity and favored an active program of neuroprotection/regeneration. We further used cortistatin-deficient mice to investigate the role of endogenous cortistatin in the control of immune responses. Surprisingly, cortistatin-deficient mice were partially resistant to EAE and other inflammatory disorders, despite showing competent inflammatory/autoactive responses. This unexpected phenotype was associated with elevated circulating glucocorticoids and an anxiety-like behavior. Our findings provide a powerful rationale for the assessment of the efficacy of cortistatin as a novel multimodal therapeutic approach to treat multiple sclerosis and identify cortistatin as a key endogenous component of neuroimmune system. The Journal of Immunology, 2013, 191: 000–000.

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Multiple sclerosis (MS) is a disabling inflammatory, autoimmune demyelinating disease of the CNS. Although the mechanisms of disease pathogenesis remain unclear, MS is considered as archetypical CD4 Th1/Th17 cell–mediated autoimmune disease in which Th1 and Th17 cells reactive to components of the myelin sheath, infiltrate CNS parenchyma, release proinflammatory cytokines and chemokines, and promote inflammatory cell infiltration and activation (1–3). Inflammatory mediators such as cytokines (i.e., IFN-γ, IL-17, and TNF-α) and free radicals, produced by infiltrating cells and resident microglia, play a critical role in demyelination, contributing to oligodendrocyte loss and axonal degeneration. Moreover, a deregulation in the mechanisms involved in maintenance of immune tolerance, especially those affecting regulatory T cells (Tregs), seems to critically contribute to establishment and progression of the autoimmune response (4). Although available therapies based on immunosuppressive agents inhibit the inflammatory component of MS and either reduce the relapse rate or delay disease onset, they do not suppress progressive clinical disability. This illustrates the need for novel multistep therapeutic approaches to prevent the inflammatory and autoimmune components of the disease and to promote mechanisms of regeneration and restoration of immune tolerance.

Cortistatin is a recently discovered neuropeptide that shows a remarkable sequential resemblance with somatostatin (5). Although it shares many functions with somatostatin, especially concerning the regulation of hormone secretion and neuronal activities (6), cortistatin exerts unique functions in the CNS and immune system. Thus, cortistatin, but not somatostatin, decreases locomotor activity, shows potent sleep-promoting activities, and deactivates inflammatory and Th1-driven responses in experimental sepsis, arthritis, and colitis (5, 7–10). Besides its release by cortical and hippocampal interneurons, cortistatin is produced by macrophages and T cells in response to inflammatory and immune stimulation (11), supporting a physiological role of cortistatin in the immune system. In this study, we investigated the potential therapeutic effect of cortistatin in two murine MS models and the role played by endogenous cortistatin in the control of inflammatory and autoimmune responses by using mice deficient for cortistatin.

Materials and Methods

Peptides and animals

Female SJL/J and C57BL/6 mice 8 wk old were obtained from Charles River. Mice lacking the gene for cortistatin (cortistatin-deficient) were generated by microinjection of Treg-resistant T cell; wt, wild-type.

Abbreviations used in this article: ACTH, adrenocorticotropic hormone; ADNP, activity-dependent neuroprotective protein; BDNF, brain-derived neurotrophic factor; CLP, cecal ligation and puncture; CRH, corticotropin-releasing hormone; CST, cortistatin; DLN, draining lymph node; DRG, dorsal root ganglion; EAE, experimental autoimmune encephalomyelitis; HP, hypothalamic-pituitary-adenal; MOG, myelin oligodendrocyte glycoprotein; MPO, myeloperoxidase; MS, multiple sclerosis; PLP, proteolipid protein; Treg, regulatory T cell; wt, wild-type.

*Institute of Parasitology and Biomedicine “López-Neyra,” Consejo Superior Investigaciones Científicas, Universidad de Granada, Spain; †Department of Psychobiology, Institute of Neuroscience, Centro de Investigación Biomédica, University of Granada, Spain; ‡Department of Pathological Anatomy, Medical School of Granada, Granada 18012, Spain; ¶Department of Molecular Biology and Biochemistry, Medical School of Cordoba, Cordoba 14071, Spain; ‡Department of Psychiatry and Behavioral Sciences, Stanford University School of Medicine, Stanford, CA 94305; and ‡Department of Biochemistry and Molecular Biology, Medical School of Sevilla, Sevilla 41009, Spain

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and for somatostatin were generated in a C57BL/6 background (12), bred in-house, and matched by sex and weight with wild-type (wt) C57BL/6 mice. All experiments with animals were performed in accordance with the European ethical guidelines and approved by the Animal Care Unit Committee from the Institute of Parasitology and Biomedicine Lopez-Neyra-Consejo Superior Investigaciones Científicas (protocol SAF2007-60101; SAF2010-16923). Proteolipid protein (PLP), HCLGKWLHPDKF) and myelin oligodendrocyte glycoprotein (MOG35–55, MEVGWYRSPFSRVHLY-RNGK) peptides were purchased from GenScript and mouse cortistatin-29 from American Peptides.

**Induction and treatment of experimental autoimmune encephalomyelitis**

To induce chronic experimental autoimmune encephalomyelitis (EAE), we immunized C57BL/6 mice s.c. with 200 μg MOG35–55 emulsified in CFA containing 400 μg Mycobacterium tuberculosis H37 RA (Difco). Mice also received i.p. injections of 200 μg pertussis toxin (Sigma) on days 0 and 2. Treatment consisted of the i.p. injection of cortistatin (1 nmol/d) or PBS (controls) for 5 consecutive days after disease onset in animals with a clinical score of 0.5–1 (onset) or of 2 (acute phase). In some animals, we applied a preemptive regimen of treatment consisting of 9 i.p. injections of cortistatin (1 nmol/d) starting 4 d before immunization. To study the role of endogenous cortistatin, we challenged CST−/− mice for EAE as described for wt C57BL/6 mice. For the relapsing-remitting EAE model, SJL/J mice also received i.p. injections of 200 ng pertussis toxin (Sigma) on days 2 PARADOXICAL ROLE OF CORTISTATIN IN AUTOIMMUNITY

**2 Determination of autoantibodies**

Spleen and DLN cells (105/ml) recovered from the C57BL/6 mice at the peak of clinical EAE (18–20 d postimmunization) were stimulated in complete medium (RPMI 1640 containing 10% FBS, 50 μM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) with 15 μM MOG35–55. Cell proliferation was evaluated by adding 2.5 μM/ml tritiated thymidine during the last 8 h of culture (72 h) and determining cpm incorporation in a Microbeta counter 1450. After 48 h, cytokine and chemokine content in culture supernatants were determined by sandwich ELISAs. We used cell activation with anti-CD3 (1 μg/ml) plus anti-CD28 (2.5 μg/ml) mAbs as controls of nonspecific polyclonal stimulation.

**Adaptive transfer of EAE**

Spleen and DLN cells recovered from untreated and cortistatin-treated C57BL/6 mice at the peak of EAE (18 d postimmunization) were stimulated in complete medium (RPMI 1640 containing 10% FBS, 50 μM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) with 15 μM MOG35–55. Cell proliferation was evaluated by adding 2.5 μM/ml tritiated thymidine during the last 8 h of culture (72 h) and determining cpm incorporation in a Microbeta counter 1450. After 48 h, cytokine and chemokine content in culture supernatants were determined by sandwich ELISAs. We used cell activation with anti-CD3 (1 μg/ml) plus anti-CD28 (2.5 μg/ml) mAbs as controls of nonspecific polyclonal stimulation.

**Histopathological analysis of EAE**

For light microscopy, cerebral and lumbar spinal cord segments were fixed with buffered 10% formalin for 48 h and processed for paraffin inclusion and sectioning. Transversal sections (4-μm thickness) were stained with Luxol fast blue, cresyl violet, and hematoxylin following the Klüver-Barrera technique (13) and were analyzed for the presence of areas of demyelination and cell infiltration using a light microscope (Olympus). For immunofluorescence staining, cerebral and lumbar spinal cord segments were fixed in 4% parafomaldehyde pH 7.4 for 4–8 h at 4 °C, treated with in 30% sucrose for 24 h, and embedded in OCT in liquid nitrogen. Transversal cryosections (10-μm thickness) were blocked with 10% PBS in PBS-T (PBS + 0.2% Triton X-100) for 30 min at 22 °C, incubated with FITC-labeled anti-CD4 mAb (2.5 μg/ml; BD Pharmingen), or PE-labeled anti-CD45 mAb (1 μg/ml; BD Bioscience), or anti-Iba1 Ab (1 μg/ml; Wako) for 18 h at 4 °C, followed by incubation of Alexa Fluor 546–labeled anti-rabbit Ab (2 μg/ml; Invitrogen). Nuclear staining was performed with Hoechst (Molecular Probes). Between steps, samples were extensively washed with PBS-T + 1% FBS. Samples were observed in a fluorescence microscope (Olympus IX81).

For immunohistochemistry, spinal cord sections were obtained as described for paraffin processing followed by blocking steps with peroxidase blocking reagents, heat-treated in 1 mM EDTA buffer pH 8 at 95 °C during 20 min for antigenic unmasking, and incubated for 30 min at room temperature with polyclonal anti-Mycelin Basic Protein Ab (Master Diagnostica). The immunohistochemical study was done on an Autostainer480 (Thermo Fisher Scientific Inc) using the polymer-peroxidase–based method and developed with dianamobenzidine. Nuclei were hematoxylin counterstained.

**Flow cytometry analysis**

For FoxP3 staining, spleen and DLN cells were isolated for C57BL/6 mice with EAE at the peak of the disease and incubated with FITC-labeled anti-CD25 and allophycocyanin-labeled anti-CD4 mAbs (4–5 μg/ml; BD Bioscience) for 8 h at 4 °C. After extensive washing, cells were fixed/permeabilized (eBioscience), stained with PE-labeled anti-FoxP3 Abs (4–5 μg/ml; eBioscience) for 30 min at 4 °C, and analyzed in a FACS Calibur flow cytometer (BD Bioscience). We used isotype-matched Abs as controls, and mouse BD Fc block to avoid nonspecific binding to FcRs.

For intracellular analysis of cytokines, spleen and DLN cells were isolated at the disease peak and stimulated at 105 cells/ml with PMA (25 ng/ml) plus ionomycin (500 ng/ml) for 8–12 h, in the presence of 3 μM monensin for the last 6 h. Cells were stained with allophycocyanin–anti-CD4 mAbs (4 μg/ml; BD Pharmingen) for 30 min at 4 °C, and analyzed in a FACS Calibur flow cytometer.

**Determination of autoantibody response**

Spleen and DLN cells (105/ml) recovered from naive C57BL/6 mice at the peak of clinical EAE (18–20 d postimmunization) were stimulated in complete medium (RPMI 1640 containing 10% FBS, 50 μM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) with 15 μM MOG35–55. Cell proliferation was evaluated by adding 2.5 μM/ml tritiated thymidine during the last 8 h of culture (72 h) and determining cpm incorporation in a Microbeta counter 1450. After 48 h, cytokine and chemokine content in culture supernatants were determined by sandwich ELISAs. We used cell activation with anti-CD3 (1 μg/ml) plus anti-CD28 (2.5 μg/ml) mAbs as controls of nonspecific polyclonal stimulation.

**Neuron and glial cell isolation and culture**

For immunocytochemistry, spinal cord sections were obtained as described for paraffin processing followed by blocking steps with peroxidase blocking reagents, heat-treated in 1 mM EDTA buffer pH 8 at 95 °C during 20 min for antigenic unmasking, and incubated for 30 min at room temperature with polyclonal anti-Mycelin Basic Protein Ab (Master Diagnostica). The immunohistochemical study was done on an Autostainer480 (Thermo Fisher Scientific Inc) using the polymer-peroxidase–based method and developed with dianamobenzidine. Nuclei were hematoxylin counterstained.

**Paradoxical Role of Cortistatin in Autoimmunity**

We used ELISA to determine the specific anti-MOG Ab responses. Maxisorb plates (Millipore) were coated overnight at 4 °C with MOG35–55 (10 μg/ml) in 0.1 M biphosphate buffer (pH 9.6), blocked with PBS/10% FBS, and incubated for 2 h at 37 °C with serial dilutions of sera obtained by cardiac puncture at the disease peak. Biotinylated anti-IgG1 or anti-IgG2a Abs (2.5 μg/ml; Serotec) were added for 1 h at 37 °C. After washing, plates were incubated with streptavidin-HRP, developed with ABTS, and absorbance was determined in a spectrophotometer.

**Neuron and glial cell isolation and culture**

Primary mixed neuron-glia, microglia, astrocytes, and oligodendrocytes were obtained from brains of newborns (postnatal days 1–3) of naive C57BL/6 and C57BL/6 mice following the protocol described for rats (14), adapted for mice. Purity of the different glial populations was determined before culture by immunofluorescence: microglia cultures were >95% Biot1, astrocyte cultures were >99% GFAP+, and cultures of oligodendrocyte precursors were >85% Olig-2+. Our neuron-glia cocultures consisted of 27.2 ± 1.8% neurons, 43.9 ± 2.4% astrocytes, and 8.9 ± 1.6% microglia (mean ± SEM). The cell cultures were used 10–14 d after the plating.

Microglia and astrocytes were cultured in DMEM/2% FBS or activated with LPS (0.1 μg/ml) or LPS (0.1 μg/ml) plus IFN-γ (50 U/ml) in the absence or presence of 100 nM cortistatin. After 24–48 h, cytokine con-

**Determination of autoantibodies**

We used ELISA to determine the specific anti-MOG Ab responses. Maxisorb plates (Millipore) were coated overnight at 4 °C with MOG35–55 (10 μg/ml) in 0.1 M biphosphate buffer (pH 9.6), blocked with PBS/10% FBS, and incubated for 2 h at 37 °C with serial dilutions of sera obtained by cardiac puncture at the disease peak. Biotinylated anti-IgG1 or anti-IgG2a Abs (2.5 μg/ml; Serotec) were added for 1 h at 37 °C. After washing, plates were incubated with streptavidin-HRP, developed with ABTS, and absorbance was determined in a spectrophotometer.
tents were determined by ELISA in supernatants, and NO production was determined by measuring oxidized nitrite amounts in culture supernatants by using the Griess reagent (8). Oligodendrocyte precursors were incubated in free-serum DMEM/Nutrient Mixture F-12 (Life Technologies) supplemented with APO-transferrin (25 μg/ml), biotin (10 nM), sodium selenite (30 nM), putrescine (1 μg/ml), insulin (5 μg/ml), hydrocortisone (20 nM), progesterone (20 nM), penicillin (100 U/ml), streptomycin (100 μg/ml), basic fibroblast growth factor (5 ng/ml), platelet-derived growth factor (5 ng/ml), and BSA (0.1%). Mature oligodendrocytes were generated by incubation of precursors with T3 hormone (30 nM) for 3–5 d. Oligodendrocyte cell death was caused by oxidative stress by incubation with 200 μM H2O2 in the absence or presence of 100 nM cortistatin, and cell survival was assayed by the reduction of MTT after 24 h of culture.

Neuronal-glial cocultures (5 × 10^5 cells/ml) were incubated in DMEM/10% FBS or stimulated with LPS (0.1 μg/ml) in the absence or presence of 100 nM cortistatin, and expression of neurotrophic factors was determined by real-time PCR as described later in RNA samples isolated after 6 h of culture.

Primary sensory neurons were isolated from lumbar dorsal root ganglia (DRGs) of 4-wk-old C57BL/6 mice as described previously (15). Isolated neurons were cultured in F12-defined medium (Invitrogen) supplemented with 10% FBS and stimulated with LPS (1 μg/ml) in the absence or presence of cortistatin (100 nM). After 24 h, NO and cytokine levels were determined in supernatants as described earlier.

RNA isolation and RT-PCR assay
We assayed gene expression of cytokine, neurotrophic factors and cortistatin by semiquantitative RT-PCR. Total RNA was isolated using TriPure (Roche) from brain and spinal cord segments. After DNase I treatment, RNA (1 μg/sample) was reverse transcribed using RevertAid First Strand cDNA synthesis kit (MBI Fermentas) and random hexamer primers. Semiquantitative PCR was performed using Taq polymerase (Biotaels) and the specific primers and conditions depicted in Supplemental Table I. Amplified PCR products were resolved in a 2% agarose gel and densitometric analysis was performed and normalized by β-actin y/o hypoxanthine guanine phosphoribosyl transferase (HPRT) expression. We also assessed actin-like myosin (myosin light chain [MLC]), and neurotrophic factor expression. We also assessed rapamycin (RAP), and brain-derived neurotrophic factor (BDNF) gene expression by real-time quantitative RT-PCR (60°C as annealing temperature) by using IQ SYBR Green Supermix (Bio-Rad) according to the manufacturer’s instructions, using β-actin for normalization and estimating fold change expression with Delta-Delta Ct method.

Induction of inflammatory models
Polynarthritis was induced in C57BL/6 wt and CST mice by s.c. immunization with 200 μg chicken type II collagen (CII; Sigma) emulsified in CFA containing 200 μg M. tuberculosis H37 RA, and s.c. boosting with 100 μg CII in CFA. Mice were injected i.p. with 50 μg LPS 26 d later to synchronize arthritis and were monitored for signs of arthritis by measuring paw swelling (measuring thickness in both hind paws with a caliper) and clinical score (0, no swelling; 1, slight swelling and erythema; 2, pronounced edema; 3, joint rigidity) in each limb.

Sepsis was induced by cecal ligation and puncture (CLP). Cecum of female CST and wt littermates. We handled the animals on alternate days 1 wk before testing and carried out the tests during the light phase of the light/dark cycle as described previously (16). In brief, mice were placed in the center of the plus maze platform and videotaped for a period of 5 min; we then determined the number of entries into and the time spent on the open arms. Moreover, mice were placed in the center of the open-field apparatus (Plexiglas box 40 × 40 × 35 cm divided in outer and inner squares) and allowed to explore the whole field for 3 min. We monitored the behavior by determining time spent in the inner and outer squares, ambulation (number of squares crossed), defecation, rearings, and time spent grooming.

Infection with Leishmania major
C57BL/6 wt and CST mice were injected s.c. in the left hind footpads with 10^4 L. major purified metacyclic promastigotes isolated from stationary cultures by negative selection with peanut agglutinin (17). Disease progression was monitored by measuring the inflammation edema with a plethysmometer (IHT Life Sciences) and the area of the cutaneous lesion of the infected footpad using a caliper, in comparison with the values obtained in the uninfected contralateral footpad. Parasite burden was determined 6 wk postinfection by the presence of amastigotes in homogenates of footpad using a limiting dilution assay (18).

Statistical analysis
All data are expressed as the mean ± SEM. Statistical analysis was carried out with two-way ANOVA followed by Bonferroni multiparameter test or Student post hoc test. We assumed significance at p < 0.05.

Results
Treatment with cortistatin reduces EAE severity
We investigated the effect of the systemic administration of cortistatin in two models of EAE that mirror different clinical characteristics of MS (19). Chronic progressive EAE induced by MOG35–55 in C57BL/6 mice is a model that mimics 20% of clinical MS. Without treatment, these mice developed moderate (27%) to severe (63%) clinical signs, and they never recovered from the disease (Fig. 1A–C). Pre-empive and delayed treatment with cortistatin after the onset or during the effector phase of the disease greatly reduced incidence and severity (Fig. 1A–D, Table I). Remarkable are the facts that most of the cortistatin-treated EAE mice displayed mild symptoms and a significant number of them completely recovered and were entirely asymptomatic 20–30 d after disease onset (Fig. 1C, 1D, Table I). Interestingly, a short treatment with cortistatin was enough to generate a long-lasting protective effect (Fig. 1).

In the majority of MS patients, clinical disease follows a relapsing-remitting course. In a model of relapsing-remitting EAE induced by PLP139–151 in SJL/J mice, a short systemic treatment with cortistatin after the onset of clinical symptoms substantially reduced clinical severity (Fig. 1E, Table I).
Cortistatin modulates the inflammatory and autoimmune components of EAE

We next investigated the mechanisms underlying the amelioration of chronic EAE after cortistatin treatment. The pathology of MS and EAE features focal areas of inflammatory infiltration and demyelination with oligodendrocyte depletion (1, 19). Histopathologic examination of spinal cords confirmed that the beneficial actions of cortistatin were due to a decrease in inflammatory infiltrates and in the subsequent demyelination and axonal loss (Fig. 2A). Evaluation of CNS infiltrates in EAE mice revealed that the inflammatory cells (CD45+) close to the perivascular area were mostly CD4+ cells and Iba1+ macrophages (Fig. 2B). Cortistatin significantly decreased the number of all these infiltrating cell populations and probably of activated Iba1+ microglia (Fig. 2B).

The reduction of inflammatory infiltration in CNS of cortistatin-treated EAE mice correlated with the expression of the decrease in expression of inflammation-related genes including IL-12, TNF-α, IL-6, IL-17, and IFN-γ, and the chemokines Rantes, MCP-1, and IP-10 (Fig. 3A, Supplemental Fig. 1A). Interestingly, despite the lower cell infiltration, cortistatin did not reduce levels of anti-inflammatory cytokines such as IL-4, TGF-β, or IL-10 in the CNS (Fig. 3A, Supplemental Fig. 1A).

In both EAE and MS, autoreactive Th1 and Th17 cells, producing IFN-γ and IL-17, respectively, infiltrate the CNS and promote the disease, whereas treatments that induce a skewing toward an IL-4–dominated Th2 response generally suppress EAE (20). Cortistatin could ameliorate EAE by reducing encephalitogenic T cell responses and/or their migration to the CNS. Therefore, we determined the proliferation and cytokine profile of peripheral T cells from cortistatin-treated EAE mice. Lymphocytes derived from DLNs of EAE mice showed marked MOG-dependent proliferation and production of IFN-γ, IL-2, and IL-17, whereas T cells from cortistatin-treated mice proliferated much less and did not produce Th1 and Th17 cytokines in the MOG-specific recall response (Fig. 3B). The effect was Ag specific because T cell activation with anti-CD3/anti-CD28 Abs resulted in similar proliferation and cytokine secretion in both groups (Fig. 3B). Moreover, cortistatin treatment did not reduce the number of effector T cells secreting IFN-γ and IL-17 in DLNs and spleen, although it elevated the percentage of IL-4–expressing CD4 cells (Fig. 3C). These results indicate that cortistatin injection during the effector phase of EAE partially inhibits autoreactive Th1 and Th17 cell activation and clonal expansion in the periphery. This effect seems to be exerted directly on lymphoid cells because cortistatin deactivated MOG-specific recall responses in vitro (Supplemental Fig. 1B). Notably, cortistatin treatment of EAE mice also reduced the capacity of activated lymphoid cells to produce GM-CSF (Fig. 3D), a cytokine mandatory for EAE induction (21).

Table I. Effect of cortistatin in chronic progressive EAE and remitting and relapsing EAE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence (%)</th>
<th>None</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
<th>Mortality</th>
<th>CDIp</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-EAE</td>
<td>Control</td>
<td>1/19 (5%)</td>
<td>1/19 (5%)</td>
<td>5/19 (27%)</td>
<td>12/19 (63%)</td>
<td>12/19 (63%)</td>
<td>97.9 ± 11.2</td>
</tr>
<tr>
<td></td>
<td>Cortistatin</td>
<td>9/18 (59%)</td>
<td>4/18 (22%)</td>
<td>3/18 (17%)</td>
<td>2/18 (11%)</td>
<td>0/18 (0%)</td>
<td>34.0 ± 6.1**</td>
</tr>
<tr>
<td>RR-EAE</td>
<td>Control</td>
<td>0/8 (0%)</td>
<td>0/8 (0%)</td>
<td>4/8 (50%)</td>
<td>4/8 (50%)</td>
<td>1/8 (12%)</td>
<td>38.4 ± 8.4</td>
</tr>
<tr>
<td></td>
<td>Cortistatin</td>
<td>5/8 (62%)</td>
<td>3/8 (37%)</td>
<td>0/8 (0%)</td>
<td>0/8 (0%)</td>
<td>0/8 (0%)</td>
<td>20.5 ± 6.6*</td>
</tr>
</tbody>
</table>

Chronic progressive EAE (CP-EAE) was induced in C57BL/6 mice by immunization with MOG35-55 and relapsing and remitting EAE (RR-EAE) was induced in SJL/J mice by immunization with PLP139-151. Immunized mice were treated i.p. for 5 d with PBS (control) or with cortistatin (1 nmol/day) starting at the onset of clinical signs (clinical score, 1). n = 3 independent experiments for CP-EAE; n = 1 experiment for RR-EAE.

*p < 0.005, **p < 0.0001 versus control.

Clinical disease index (CDI), the mean of the sum of the daily disease scores.
High levels of circulating Abs directed against myelin Ags invariably accompany the development of MS and EAE, and are major factors in determining susceptibility to the disease (3). Cortistatin regulated serum levels of MOG-specific IgG, particularly reducing the ratio between IgG2a and IgG1, generally reflective of Th1 and Th2 activities, respectively (Fig. 3E). Cortistatin induces the emergence of functional Tregs in EAE

We next evaluated the capacity of cortistatin to generate Tregs during EAE, because Tregs confer significant protection against EAE by promoting Th2 protective responses and deactivating autoreactive T cells and their homing to CNS (22, 23). We found that cortistatin injection increased the percentage of IL-10–secreting CD4 cells and CD4+CD25+Foxp3+ Treg in DLNs and spleens of mice suffering from EAE (Fig. 3F). This increase in Tregs could be functionally related with the suppression of encephalogenic responses and the induction of immune tolerance, because injection of T cells from spleen/DLNs of cortistatin-treated, but not of untreated EAE mice, into diseased mice alleviated their clinical signs (Fig. 3G). Experiments of cell depletion before transference suggested that the capacity to generate tolerance resides in the CD4+CD25+ Treg population (Fig. 3G, right panel). Moreover, injection of CD4+CD25+ T cells isolated from cortistatin-treated EAE mice into diseased mice significantly protected from EAE development, being more efficient cell by cell than CD4+CD25+ T cells isolated from untreated EAE mice (Supplemental Fig. 1C).

Cortistatin regulates CNS resident glial cells and promotes neuroprotective responses

We next investigated whether, besides its immunoregulatory activity, cortistatin exerts an active protective effect in the CNS directly acting on resident cells. We observed that cortistatin treatment significantly increased the expression of neuroprotective...
Cortistatin affects Th1, Th17, and Th2 numbers in lymphoid organs. Treatment with cortistatin increased ADNP levels in spleen/DLNs of EAE mice, mainly on MOG recall responses (Fig. 4B, left middle panels). This effect could be mediated directly on T cells because cortistatin elevated ADNP expression in spleen/DLN cell cultures (Fig. 4B, right panel). Contrary to ADNP, we did not detect BDNF in spleen/DLNs of untreated or cortistatin-treated EAE mice (data not shown). Supporting its effect at the CNS level, cortistatin increased the expression of ADNP and BDNF in neuron-glia cocultures in both basal and inflammatory conditions (Fig. 4C).

Local production of cytotoxic factors by activated microglia and astrocytes in an inflammatory milieu critically contributes to the patholgy of MS and EAE by inducing demyelination, oligodendrocyte loss, and axonal degeneration (1, 3). Cortistatin reduced the production of inflammatory cytokines such as TNF-α, and of IL-6 and NO by activated macroglia and microglia, respectively (Fig. 4D). However, cortistatin did not affect significantly the production of inflammatory mediators by LPS-activated primary mouse DRG neurons (Fig. 4D) and rat PC12 neurons (data not shown). Notably, cortistatin decreased cell death induced by oxidative stress in precursor and mature oligodendrocytes (Fig. 4E). These data suggest that cortistatin could promote protective responses in EAE by inducing neurotrophic factors at both central and peripheral levels, by downregulating the destructive inflammatory response mediated by resident glial cells and directly avoiding oligodendrocyte loss.

Paradoxical effect of cortistatin deficiency in EAE and other inflammatory disorders

Once the protective action of cortistatin in EAE was established, we asked about the role played by endogenous cortistatin in the regulation of inflammatory and autoimmune responses in EAE. We found that the expression of cortistatin expression in the CNS of mice inversely correlated to the clinical severity of EAE (Fig. 5). As expected, macrophages, microglia, and lymphocytes deficient in cortistatin responded with exacerbated responses to inflammatory and T cell stimulation (Fig. 6A; Supplemental Fig. 2A), supporting an autocrine/paracrine effect of cortistatin. Surprisingly, the induction of EAE in CST−/− mice showed a paradoxical response. CST−/− mice challenged for the induction of EAE, showed delayed disease onset (11.7 ± 0.8 d for wt versus 15.3 ± 1.1 d for CST−/−, p < 0.05) and reduced clinical disease index (CDI) (CDI: 79.2 ± 10.3 for wt versus 39.9 ± 6.4 for CST−/−, p < 0.005) compared with wt mice (Fig. 6B). Moreover, CST−/− mice showed less demyelination and spinal cell infiltration than wt mice, which correlated with decreased CNS expression of inflammatory mediators, especially chemokines, and elevated neurotrophic factors and Th2 cytokines in these animals (Supplemental Fig. 2B–D). However, mice that lacked somatostatin, a peptide structurally related with cortistatin, experienced development of exacerbated EAE signs (Supplemental Fig. 2E).

We next investigated whether the unexpected effect of cortistatin deficiency in the development of EAE was due to a defect in the autoreactive response. CST−/− mice generated similar levels of autoantibodies than wt mice, and immune cells from CST−/− mice with EAE strongly responded to MOG restimulation, even with higher Th1 and Th17 autoreactive responses (Fig. 6C; Supplemental Fig. 2F, 2G). The encephalitogenic capacity of T cells of CST−/− mice was confirmed in experiments of adoptive transfer of EAE to naive mice. Transfer of spleen/DLN cells from CST−/− mice restimulated ex vivo with MOG caused an EAE more severe and with an earlier onset in the recipient mice than ADNP) may be produced by resident cells (neurons and glia) and infiltrating T cells (26), we investigated whether cortistatin exerted this effect at central and/or peripheral levels. Treatment with cortistatin increased ADNP levels in spleen/DLNs of EAE mice, mainly on MOG recall responses (Fig. 4B, left middle panels). This effect could be mediated directly on T cells because cortistatin elevated ADNP expression in spleen/DLN cell cultures (Fig. 4B, right panel). Contrary to ADNP, we did not detect BDNF in spleen/DLNs of untreated or cortistatin-treated EAE mice (data not shown). Supporting its effect at the CNS level, cortistatin increased the expression of ADNP and BDNF in neuron-glia cocultures in both basal and inflammatory conditions (Fig. 4C).

Factors, such as BDNF and activity-dependent neuroprotector protein (ADNP), in the CNS of EAE mice (Fig. 4A), which are involved in processes of remyelination, axonal growth, and neuroregeneration (24–26). Because neurotrophic factors (especially ADNP) may be produced by resident cells (neurons and glia) and infiltrating T cells (26), we investigated whether cortistatin exerted this effect at central and/or peripheral levels. Treatment with cortistatin increased ADNP levels in spleen/DLNs of EAE mice, mainly on MOG recall responses (Fig. 4B, left middle panels). This effect could be mediated directly on T cells because cortistatin elevated ADNP expression in spleen/DLN cell cultures (Fig. 4B, right panel). Contrary to ADNP, we did not detect BDNF in spleen/DLNs of untreated or cortistatin-treated EAE mice (data not shown). Supporting its effect at the CNS level, cortistatin increased the expression of ADNP and BDNF in neuron-glia cocultures in both basal and inflammatory conditions (Fig. 4C).

Cortistatin alleviates EAE severity by modulating both inflammatory and autoimmune components of the disease. Mice with MOG35–55–induced chronic EAE were treated with PBS (control) or cortistatin for 5 d at the disease onset. (A) Expression of inflammatory cytokines and chemokines determined by RT-PCR in spinal cords at the disease peak (6/group, n = 2). (B) Cortistatin decreases peripheral encephalitogenic Th1/Th17 responses. Proliferation and cytokine production by DLN cells isolated at EAE peak and stimulated with medium, the encephalitogenic Ag (MOG35–55), or a polyclonal stimulus (anti-CD3/CD28 Abs). We obtained similar results with spleen cells (6/group, n = 2). (C) Cortistatin affects Th1, Th17, and Th2 numbers in lymphoid organs. Spleen cells isolated at EAE peak were assayed for intracellular cytokine expression by flow cytometry in the CD4 population. We used naive mice as basal controls. We obtained similar results with DLNs (6–8/group, n = 3). (D) Cortistatin reduces GM-CSF production upon restimulation (6/group, n = 2). (E) MOG-specific IgG1 and IgG2a levels in sera collected at the disease peak (14/group, n = 3). (F) Cortistatin induces the emergence of Tregs in EAE. Percentage of CD4+IL-10+ and CD4+CD25+Foxp3+ Tregs in DLN cells isolated at EAE peak (18–20/group, n = 3). (G) Treatment (arrow) of EAE mice with T cells or CD25-depleted CD4 cells isolated from spleen/DLNs of EAE mice that were previously treated with PBS (control) or cortistatin (7–9/group, n = 2). We used untreated EAE mice as reference. *p < 0.05, **p < 0.005, ***p < 0.0001 versus control.
that induced by transfer of cells from wt mice (Fig. 6D). Interestingly, recipient CST<sup>−/−</sup> mice were mostly resistant to adoptive transfer of EAE with encephalitogenic cells, suggesting that the effector phase of the disease is affected (Fig. 6D).

We further studied whether this paradoxical effect observed in CST<sup>−/−</sup> mice was exclusive of EAE or it could be extended to other inflammatory conditions, in which cortistatin-based therapies were proved effective (8–10). CST<sup>−/−</sup> mice showed delayed disease onsets and initial lower severity in collagen-induced polyarthritis and experienced less severe chronic colitis than wt mice (Fig. 6E, Supplemental Fig. 3A). Similarly, lack of cortistatin that induced by transfer of cells from wt mice (Fig. 6D). Interestingly, recipient CST<sup>−/−</sup> mice were mostly resistant to adoptive transfer of EAE with encephalitogenic cells, suggesting that the effector phase of the disease is affected (Fig. 6D).

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paradoxical role of cortistatin in autoimmunity

temic injections of the glucocorticoid receptor antagonist RU-486 partially reversed the phenotype observed in CST−/− mice in both conditions (Fig. 7D). However, pre-emptive cortistatin injection did not reverse this resistance, probably because cortistatin treatment is itself protective (Supplemental Fig. 3E).

Discussion

The initial stages of MS and EAE involve multiple steps that can be divided into two main phases: early events associated with initiation and establishment of autoimmunity to myelin sheath components, and later events associated with the evolving destructive inflammatory responses. Progression of the autoimmune response involves the development of reactive Th1 and Th17 cells with encephalitogenic potential, their entry into the CNS, and further recruitment of inflammatory cells through multiple mediators (1, 3). In this study, we report that the neuropeptide cortistatin provides a highly effective therapy for chronic and relapsing-remitting EAE. The therapeutic effect of cortistatin is associated with a striking reduction of the two deleterious components of the disease, namely, the autoimmune and inflammatory responses. As a consequence, cortistatin reduced the appearance of inflammatory infiltrates in the CNS and the subsequent demyelination and axonal damage typical of EAE.

Our data indicate that treatment with cortistatin decreased the presence of encephalitogenic Th1 and Th17 cells in the periphery and CNS. This effect is mostly exerted by regulating the encephalitogenic sensitization in the peripheral immune compartment. Importantly, this treatment did not result in a general immunosuppression. Cortistatin did not affect the number of Th1 and Th17 cells in lymphoid organs or the response to a polyclonal stimulation, but it specifically impaired the activation of MOG-specific Th1 and Th17 responses; at the same time, it favored...
Th2 responses, which is reflected by a class switch in autoantibodies. The potent suppressive effect of cortistatin on the activation of encephalitogenic Th17 cells might be an important component in its protective effect on EAE, because of the critical role that Th17 cells play in the disease effector phase in EAE and MS (28, 29). Of relevance is also the fact that cortistatin downregulated the production of GM-CSF by activated T cells, because it serves a nonredundant function in the initiation of autoimmune inflammation in EAE regardless of Th cell polarization (21).

The increase in the repertory of CD4+CD25+Foxp3+ Tregs in lymphoid organs could partially explain the specificity of Ag of the long-lasting protective response generated by cortistatin treatment, and that cortistatin injection inhibits events in the inflammatory phase of EAE after the activation/differentiation of Ag-specific effector Th1/Th17 cells. In agreement with our results, a recent study described the generation of CD4+CD25+ Tregs by cortistatin in a model of allogeneic skin transplantation (30). However, to our knowledge, our work is the first to demonstrate the involvement of functional Tregs in the therapeutic action of cortistatin in autoimmunity. Further investigation will determine whether this increase in Tregs is a consequence of an effect of cortistatin on the expansion of already existing Tregs or on de novo generation of peripheral Tregs. Other neuropeptides, such as vasoactive intestinal peptide, urocortin, and adrenomedullin, with protective effects in autoimmunity (31), increased the repertory of Tregs by promoting their generation from the non-Treg compartment through direct actions on T cells and indirectly on tolerogenic dendritic cells (31). It remains also unknown whether the population of IL-10–secreting CD4 T cells increased by cortistatin corresponds to a subtype of Tr1-like cells (32) and whether it plays any role in its protective effect in EAE.

Regarding the inflammatory response in EAE, it is evident that the regulation of a wide spectrum of inflammatory mediators by cortistatin could suppose an advantage over other therapies directed against a single mediator. The reduction in the inflammatory infiltration of CNS observed in the cortistatin-treated EAE mice seems to be associated with the decrease in the levels of chemokines in CNS parenchyma. This is especially relevant for chemokines such as IP-10 (chemotactic for Th1 cells), Rantes (for T cells), and MCP-1 (for macrophages and T cells) that contribute to MS neuropathology (1, 3). Interestingly, cortistatin did not decrease MDC-1, which is chemotactic for Th2 cells. As a consequence, cortistatin increased the rate of Th2 versus Th1/Th17 cytokines in the CNS of EAE mice, which is protective for the disease. Whether the effect on chemokines is exerted at the local level or as a consequence of the peripheral action of cortistatin (i.e., on Th17 or GM-CSF) is unknown. The fact that cortistatin failed to inhibit Rantes secretion by activated glial cells in vitro argues against a local effect of cortistatin in the chemokine repertoire. However, we found that cortistatin might exert its anti-inflammatory action locally by downregulating the production of cytokines and NO by astrocytes and microglia. This effect on resident inflammatory cells, together with the inhibitory action of cortistatin on infiltrating macrophages (9), probably contributes to the protection against oligodendrocyte/neuronal loss and axonal damage in this inflammatory milieu.

However, attention has recently focused on regenerative mechanisms as targets for therapy in MS, especially in the secondary progressive phase of the disease. Notably, the delayed treatment with cortistatin induced a whole recovery in a significant number of animals, suggesting a role of cortistatin in repair, neuroregeneration, or both. In this article, we found that cortistatin protected oligodendrocytes from cell death in an oxidative milieu as occurs in EAE. Moreover, cortistatin increased the local levels of BDNF and ADNP, which induce axonal outgrowth, remyelination, and rescue of degenerating neurons (24, 25). For ADNP, this effect is exerted on both peripheral lymphoid cells and resident CNS cells. However, the increase in BDNF could mainly depend on a central action of cortistatin on neuron-glial cells.
Which is the role of endogenous cortistatin in this scenario? Evidence suggests that cortistatin is an endogenous immunomodulator: immune cells produce cortistatin in response to inflammatory/immune stimulation (11); cortistatin produced by macrophages and lymphocytes plays an autocrine/paracrine regulatory role in the immune response, as lack of cortistatin predisposes to stronger response to immunostimulation (Fig. 6A); an inverse correlation exists between EAE severity and the levels of cortistatin in the CNS (Fig. 5); and a deficiency of cortistatin in the retina of diabetic patients with retinopathy correlated with increased retinal neurodegeneration and glial activation (33). These findings suggest that endogenous cortistatin might normally provide protection against autoimmune and neurodegenerative pathologies. Indeed, we found exacerbated local inflammatory responses in CST \(-/-\) mice. However, lack of cortistatin surprisingly conferred certain resistance to EAE and other systemic inflammatory pathologies, with delayed onset and mild clinical profile. Despite this, the levels of autoantibodies and Ag-rechallenge experiments indicated that MOG-treated CST \(-/-\) mice exhibited robust Th1/Th17 cell responses. Moreover, adoptive transfer of the disease with lymphocytes from immunized CST \(-/-\) mice to naive recipients supports their encephalitogenic potential. This suggests a compensatory mechanism in CST \(-/-\) mice other than an intrinsic defect in immune cells. Although unpredictable, the case of cortistatin is not unique. Mice that lacked the immunomodulatory neuropeptide vasoactive intestinal peptide were almost completely resistant to EAE (34), although they generate Th1 with stronger encephalitogenic capacity than wt mice.

We present evidence that supports the partial involvement of an altered glucocorticoid system in the paradoxical phenotype observed in CST \(-/-\) mice challenged through induction of EAE and other pathologies. Glucocorticoids are well-known immunosuppressive factors with widespread actions in different components of the immune system. The elevated levels of corticosterone found in CST \(-/-\) mice could partially explain their resistance to systemic immune responses. These abnormal glucocorticoid levels might be a consequence of an altered HPA axis (i.e., elevated adrenal corticotropic hormone [ACTH] levels) (12), which is reflected by an exacerbated anxiety-like behavior in these animals. Numerous studies correlate stress to states of immunosuppression and alteration in the HPA axis (27). Indeed, CST \(-/-\) mice were more susceptible to infection by the intracellular parasite L. major, which depends on fully functional Th1-driven inflammatory responses to be eliminated. How cortistatin regulates this neuroimmune axis is still unknown. However, evidence indicates that cortistatin could act at multiple levels as an endogenous brake for immune responses in CST \(-/-\) mice.

Moreover, the anti-inflammatory ghrelin and the proinflammatory prolactin are elevated and decreased, respectively, in CST \(-/-\) mice (12). Besides a direct effect on the immune response (41), an elevated ghrelin level could affect the activity of HPA axis in CST \(-/-\) mice because ghrelin stimulates the production of hypothalamic CRH and pituitary ACTH (42, 43).

In summary, we are proposing a novel treatment strategy for MS that is targeted to the inhibition of the different neuropathological components of the disease, whereas restoring long-lasting immune tolerance and mounting an active program of neuroprotection. This multimodal action would suppose a therapeutic advantage versus current treatments. Its ability, on delayed administration, to ameliorate the ongoing disease also fulfills an essential prerequisite for a therapeutic agent for MS. This work also demonstrates that cortistatin from the immune source plays a critical role in the tuning of the immune responses in health and disease, and that cortistatin is a key player in the bidirectional communication that exists between the neuroendocrine and immune systems, which altogether define the final immune response of our body.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Table I. Primers and temperatures of annealing used in the semiquantitative RT-PCR assays.

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Supplemental Figure Legends

Supplemental Figure 1. Mechanisms involved in the therapeutic action of cortistatin in EAE. (A) Cortistatin modulates the content of inflammatory mediators in the CNS of EAE mice. Mice with MOG\textsubscript{35-55}-induced chronic EAE were treated with PBS (control) or cortistatin for 5 d starting at the onset of disease. Proteins were purified from spinal cords and brains harvested at the peak of clinical disease and the expression of inflammatory cytokines and chemokines was determined by ELISA. 5-6/group, n=2. (B) Cortistatin directly deactivates encephalitogenic T-cell responses \textit{in vitro}. Pooled spleen and DLN cells isolated from mice suffering chronic EAE at the peak of disease were incubated with medium or restimulated with MOG in the absence (control) or presence of cortistatin (100 nM) and the proliferative response and cytokine levels in the cultures were determined. 10/group, n=2. (C) Treatment (arrow) of EAE mice (6/group, n=2) with CD4\textsuperscript{+}CD25\textsuperscript{+} cells isolated from spleen/DLN of EAE mice that were previously treated with PBS (control) or cortistatin. We used untreated EAE mice as reference. \*p<0.05; \***p<0.0001 vs control untreated EAE mice.

Supplemental Figure 2. Mice deficient for cortistatin show competent encephalitogenic responses. (A) Microglia deficient in cortistatin showed exacerbated inflammatory responses. Microglia isolated from C57BL/6 wt and CST\textsuperscript{-/-} mice were stimulated with LPS and the levels of NO and cytokines determined in culture supernatants after 24 h of culture (n=3 experiments, in duplicates). (B) Chronic EAE was induced by immunization with MOG in wt and CST\textsuperscript{-/-} mice (5/group) and histopathological analysis was performed in transversal spinal cord sections isolated at day 23. (C) Chronic EAE was induced by MOG immunization in wt and CST\textsuperscript{-/-} mice (6/group, n=2) and the expression of inflammatory cytokines and chemokines
determined by RT-PCR from spinal cords at the disease peak and expressed relative to EAE wt mice (dashed line). (C) Chronic EAE was induced in wt and CST^{−/−} mice (4/group, n=2) and the expression of ADNP and BDNF determined by RT-PCR in mixed brain/spinal cord samples isolated 15 d and 23 d post-immunization. Results are expressed as fold changes relative to samples obtained from naïve mice (dashed line). (E) Mice deficient for somatostatin (SOM^{−/−}) developed exacerbated chronic EAE in comparison to wt mice (4/group). (F) Levels of MOG-specific Abs in sera isolated from wt and CST^{−/−} mice (4/group) 15 d and 23 d after EAE induction. (G) Production of cytokines of DLN cells isolated at day 23 from wt and CST^{−/−} mice (4/group) suffering chronic EAE re-stimulated with MOG_{35-55}. Similar results were obtained with spleen cells. *p<0.05, **p<0.001 vs wt mice.

Supplemental Figure 3. Cortistatin deficiency differentially affects to systemic and local inflammatory responses. (A) Incidence and clinical score of collagen-induced arthritis in wt and CST^{−/−} mice (10-14/group). Paw edema was measured at day 35. (B) Sepsis was induced in wt and CST^{−/−} mice (6/group) by CLP, and 24 h later, cytokine and chemokine contents in serum (peritoneal suspension showed similar results) were assayed by ELISA and lungs were processed in paraffin, sectioned and stained with H&E for histopathological analysis. (C) Peritoneal macrophages isolated 2 d after CLP were cultured in complete medium for 48 h and the spontaneous cytokine release was measured in supernatants by ELISA (3/group, in duplicate). (D) Induction of local inflammation by intraplantar injection of carrageenan in wt and CST^{−/−} mice. Wt mice were treated intraplantarly with cortistatin (2 nmol) immediately after carrageenan. After 4 h, edema was determined by the increase in the inflamed paw volume (versus basal, BL) and the infiltration of neutrophils measured as MPO activity. The content of
inflammatory cytokines and chemokines in the inflamed paw was measured 2 h after carrageenan injection. n=6-8/group, two independent experiments. (E) Cortistatin did not reverse the response of CST−/− mice to EAE and sepsis. Cortistatin was injected i.p. (1 nmol) for 9 d starting 4 d before MOG-immunization (left panel, 8-9/group), or at -2, 0, 8, 18 and 24 h respect to CLP intervention (right panel, 8-12/group). *p<0.05, **p<0.005, ***p<0.0001 vs wt mice.
Supplemental Figure 1

A) [Graph showing cytokine levels (ng/mg tissue) for control and cortistatin treatment.]

B) [Graph showing proliferation (cpm x 10^3) for control and cortistatin treatment.]

C) [Graph showing mean clinical score for untreated EAE mice, CD4^+ CD25^- control, and CD4^+ CD25^- cortistatin treatment over days postimmunization.]
Supplemental Figure 2
Supplemental Figure 3