Cortistatin, an antiinflammatory peptide with therapeutic action in inflammatory bowel disease

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Cortistatin is a recently discovered cyclic neuropeptide related to somatostatin that has emerged as a potential endogenous anti-inflammatory factor based on its production by, and binding to, immune cells. Crohn’s disease is a chronic debilitating disease characterized by severe T helper 1 (Th1)-driven inflammation of the gastrointestinal tract. The aim of this study is to investigate the therapeutic effect of cortistatin in a murine model of colitis. Cortistatin treatment significantly ameliorated the clinical and histopathologic severity of the inflammatory colitis, abrogating body weight loss, diarrhea, and inflammation and increased the survival rate of the colitic mice. The therapeutic effect was associated with down-regulation of inflammatory and Th1-driven autoimmune response, including the regulation of a wide spectrum of inflammatory mediators. In addition, a partial involvement of regulatory IL-10-secreting T cells in this therapeutic effect was demonstrated. Importantly, cortistatin treatment was therapeutically effective in established colitis and avoided the recurrence of the disease. This work identifies cortistatin as an antiinflammatory factor with the capacity to deactivate the intestinal inflammatory response and restore mucosal immune tolerance at multiple levels. Consequently, cortistatin represents a multistep therapeutic approach for the treatment of Crohn’s disease and other Th1-mediated inflammatory diseases.

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Inflammatory bowel disease (IBD) is a family of chronic, idiopathic, relapsing, and tissue-destructive diseases characterized by dysfunction of mucosal T cells, altered cytokine production, and cellular inflammation that ultimately leads to damage of the distal small intestine and the colon mucosa (1). IBD is clinically subdivided into two phenotypes: Crohn’s disease (CD) and ulcerative colitis. CD is an incurable autoimmune disease with a prevalence of 0.05% that leads to chronic inflammation resulting in a range of gastrointestinal and extraintestinal symptoms, including abdominal pain, rectal bleeding, diarrhea, weight loss, skin and eye disorders, and delayed growth and sexual maturation in children (2). Although its etiology remains unknown, there is circumstantial evidence to link CD to a failure of the mucosal immune system to attenuate the immune response to endogenous antigens (1). Several animal models of CD have been developed lately. Although many of these models incompletely resemble the human disease (3, 4), the hapten-induced model of colonic inflammation, in which 2,4,6-trinitrobenzene sulfonic acid (TNBS) is delivered intrarectally, displays human CD-like clinical, histopathological, and immunological features (4). In this model, intestinal inflammation results from a covalent binding of the haptenizing agent to autologous host proteins with subsequent stimulation of a delayed-type hypersensitivity to TNBS-modified self-antigens. Similarly to human CD, TNBS-induced colitis is marked by an immune response to exaggerated gut-associated lymphoid tissue development, which gives rise to a prolonged severe transmural inflamed intestinal mucosa characterized by uncontrolled production of inflammatory cytokines and oligoclonal expansion and activation of CD4 T cells specifically associated with a T helper 1 (Th1) response (4).

Therapeutic agents currently used for CD are not entirely effective, are rather nonspecific, and have multiple adverse side effects (2). In most cases, surgical resection is the ultimate alternative. Therefore, the present therapeutic strategy is to find drugs or agents that specifically modulate both components of the disease, i.e., the inflammatory and the Th1-driven responses.

Cortistatin is a recently discovered cyclic neuropeptide related to somatostatin (5). Cortistatin binds to all five cloned somatostatin receptors and shares many of the somatostatin pharmacological and functional properties, including the depression of neuronal activity and inhibition of cell proliferation (6). However, cortistatin also has many properties distinct from somatostatin, including induction of slow-wave sleep and reduction of locomotor activity (6). Cortistatin, but not somatostatin, has been detected in various human immune cells, including lymphocytes, monocytes, macrophages, and dendritic cells (7, 8). Some of the somatostatin immuno-modulatory actions (9) with a predominant antiinflammatory effect might be shared by cortistatin. Also, because cortistatin expression levels correlate with immune cell differentiation and activation (7, 8), cortistatin might be a major endogenous regulatory factor in the immune system. In addition to the binding to somatostatin receptors in immune cells, cortistatin can also bind to other receptors, including the receptor for the growth hormone secretagogue ghrelin (10), a hormone recently described as a potent antiinflammatory factor (11, 12). The aim of this study is to investigate the potential antiinflammatory action of cortistatin and its potential therapeutic use in the TNBS-induced model of colitis.

Results

Cortistatin Inhibits the Production of Inflammatory Mediators by Activated Macrophages in Vitro. To investigate the potential antiinflammatory action of cortistatin, we evaluated first the effect of cortistatin on the production of several inflammatory mediators by peritoneal macrophages. Cortistatin inhibited the production of TNF-α, IL-6, IL-1β, IL-12, macrophage-inflammatory protein (MIP)-2, Rantes, and nitric oxide (NO) by activated macrophages (Fig. 5, which is published as supporting information on the PNAS web site). Interestingly, cortistatin showed a higher inhibitory effect than the structurally related peptide somatostatin, and the somatostatin receptor-antagonist cyclosomatostatin partially reversed the inhibitory effect of cortistatin, although it fully blocked the effect of somatostatin (Fig. 5). These results suggest that cortistatin could exert its effects through both somatostatin receptor-dependent and -independent mechanisms. Indeed, a ghrelin-

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Abbreviations: CD, Crohn’s disease; IBD, inflammatory bowel disease; LPMC, lamina propria mononuclear cell; MIP, macrophage-inflammatory protein; MLN, mesenteric lymph node; MPO, myeloperoxidase; SAβA, serum amyloid A; Th1, T helper 1; TNBS, 2,4,6-trinitrobenzene sulfonic acid; Treg, regulatory T cell.

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receptor antagonist partially blocked the inhibitory effect of cortistatin (Fig. 5).

**Cortistatin Protects Against TNBS Colitis Development.** We next investigated the potential therapeutic action of cortistatin in the TNBS model of colitis. Mice subjected to intrarectal administration of TNBS in 50% ethanol developed a severe illness characterized by bloody diarrhea, rectal prolapse, pancolitis accompanied by extensive wasting syndrome, and a profound and sustained weight loss resulting in a mortality of 60% (Fig. 1A–D). Mice treated with cortistatin 12 h after TNBS instillation rapidly recovered the lost body weight, improved the wasting disease, and had a healthy appearance, with a survival of 90%, similar to control mice treated with 50% ethanol alone (Fig. 1A–D). The therapeutic effect of cortistatin was dose-dependent, showing maximal effects at doses between 0.2 and 2 nmol (25–250 μg/kg) (Fig. 1B). Microscopic examination of colons obtained 3 and 7 days after colitis induction showed striking hyperemia, inflammation, and necrosis compared with control animals that only showed slight inflammation (Fig. 1E). In contrast, the colons of cortistatin-treated mice showed no signs of macroscopic inflammation (Fig. 1E). Histological examination of the distal colon of mice given TNBS showed transmural inflammation involving all layers of the bowel wall with a marked increase in the thickness of the muscular layer, adherence to surrounding tissues, patchy ulceration, epithelial cell loss, pronounced depletion of mucin-producing goblet cells, reduction of the density of the tubular glands, disseminated fibrosis, and focal loss of cripts (Fig. 1F). Inflammatory cell infiltrates consisted of macrophages, lymphocytes, and neutrophils in the lamina propria (Fig. 1F), along with enlargements of lymphoid follicles in the colon (data not shown). Immunohistological analysis revealed CD4 T cells, TNF-α-producing cells, and CD11b+ cells, i.e., granulocytes and macrophages (Fig. 1G). Neutrophil infiltration correlated with increased colonic myeloperoxidase (MPO) activity (Fig. 1H). When mice were treated with cortistatin, a striking improvement of these macroscopic and histological signs became apparent, with a significant reduction in the inflammatory activity and neutrophil infiltration (Fig. 1F–H).

We next investigated whether cortistatin would be effective during the later phases of the disease with colitis fully established. Administration of cortistatin on 3 consecutive days starting 6 days after onset of disease rapidly reversed the lost body weight (Fig. 2A). In addition, we examined whether cortistatin was able to prevent disease recurrence. TNBS-treated mice reexposed on day 9 to a second dose of TNBS rapidly died (100% mortality) due to severe colitis and body weight loss. Mice receiving an unique dose of
Cortistatin treatment abrogates established colitis and reduces disease recurrence. (A) Established colitis. Colitis was induced by intracolonic administration of TNBS (1.5 mg per mouse) at days 0 and 6. Mice were treated daily for 3 consecutive days with cortistatin (2 nmol per mouse) starting 6 days after TNBS administration (arrow). Disease progression was assessed by body weight loss. *n = 8 mice per group. (B) Disease recurrence. Colitis was induced in BALB/c and SJL mice by intracolonic administration of TNBS (3 mg per mouse) at days 0 and 9 (arrows). Mice were treated i.p. with cortistatin (2 nmol per mouse) 12 h after TNBS injection. Controls were given with a second injection of ethanol on day 9. Disease progression was assessed by body weight loss and survival percentage. The numbers in parentheses represent daily mortality percentages after the second TNBS infusion. *n = 8–10 mice per group.

cortistatin 12 h after the initial colitis induction survived and did not suffer disease recurrence after a second administration of TNBS (Fig. 2B). The therapeutic effect of cortistatin was confirmed in SJL mice, a murine strain more susceptible for TNBS-induced colitis (Fig. 2B).

Cortistatin was more efficient at ameliorating the body weight loss and colitis than its two structurally related peptides, somatostatin- and ghrelin-receptor antagonists partially reversed cortistatin’s effects (Fig. 6, which is published as supporting information on the PNAS web site).

**Discussion**

The present study reports cortistatin as a peptide with potent antiinflammatory actions *in vivo*. Our data demonstrate that cortistatin provides a highly effective treatment for TNBS-induced colitis, a murine experimental model of CD. A single injection of cortistatin at the onset of the disease ameliorated the clinical and inflammatory conditions.

**Cortistatin Suppresses Th1 Cytokine Response and Stimulates IL-10 Production in TNBS-Induced Colitis.** Although macrophages and neutrophils are the major sources of inflammatory mediators, CD4 T cells play a key role in the initiation and perpetuation of CD by producing IFN-γ, a potent inducer of the inflammatory response (4). In fact, CD and TNBS-induced colitis are considered Th1-type cell-mediated autoimmune diseases (4, 13, 14). Therefore, we determined the effect of the cortistatin treatment of TNBS-induced colitis on the ability of LPMC and draining mesenteric lymph node (MLN) cells to produce IFN-γ and proliferate *in vitro*. LPMC and MLN cells obtained from TNBS-treated mice proliferate more and produced significantly more IFN-γ than ethanol-treated mice, and *in vitro* activation of these cells caused further cell expansion and increased amounts of IFN-γ (Fig. 4A and B). In contrast, LPMC and MLN cells isolated from cortistatin-treated colitic animals proliferate less and produce significantly lower amounts of IFN-γ than do cells isolated from TNBS-treated mice, even after potent T cell stimulation (Fig. 4A and B). In addition, the production of the regulatory cytokine IL-10 was significantly increased in LPMC and MLN cells obtained from cortistatin-treated mice, specially upon *in vitro* activation; the Th2-type cytokine IL-4 was not significantly affected (Fig. 4B). Thus, cortistatin decreased Th1 cytokine production *in vivo* and abrogated the responsiveness of LPMC and MLN cells to subsequent *in vitro* stimulation. The decreased IFN-γ production and proliferation of LPMC and MLN cells is specific to cells residing in the lamina propria environment or draining MLN, because splenocytes from cortistatin-treated and untreated TNBS mice equally proliferate and produce IFN-γ upon stimulation (data not shown). Given that the decrease in IFN-γ production induced by cortistatin treatment could be a consequence of either down-regulation of IFN-γ release or inhibition of Th1 cell differentiation and that the production of IL-10 could be due to macrophages and CD4 T cells, we determined the intracellular expression of these cytokines by flow cytometry in sorted CD4 T cells. Cortistatin significantly decreased the number of IL-2/IFN-γ-producing Th1 cells and increased the number of IL-10-producing CD4 T cells in LPMC and MLN (Fig. 4B). Thus, cortistatin administration to colitic mice regulates the generation/differentiation of autoreactive/inflammatory Th1 cells and, presumably, regulatory IL-10-secreting T cells.

**Treatment with Cortistatin Reduces Systemic and Mucosal Inflammatory Responses in Mice with TNBS-Induced Colitis.** We next evaluated the effect of cortistatin on the production of inflammatory mediators that are mechanistically linked to TNBS-induced colitis. Cortistatin dramatically reduced protein and mRNA expression of inflammatory cytokines (TNF-α, IFN-γ, IL-6, IL-1α, IL-1β, IL-12, IL-18, IL-17, IL-15, and macrophage migration inhibitory factor), chemokines (Rantes, MIP-1α, MIP-1β, MIP-3β, monocyte chemotactic proteins 1 and 3, IP-10, and MIP-2) and chemokine receptors (CCR-1, CCR-2, CCR-3, CCR-5, and CCR-7) in the mucosa of colitic mice (Fig. 3). In addition, colons of cortistatin-treated mice showed increased levels of the antiinflammatory cytokine IL-10 and chemokine receptors CCR-4 and CCR-8 (Fig. 3). The decrease in inflammatory mediators could be a consequence of the diminished infiltration of inflammatory cells in the colonic mucosa in the cortistatin-treated colitic mice. However, lamina propria mononuclear cells (LPMC) isolated from cortistatin-treated mice produced lower levels of proinflammatory factors (TNF-α, IL-6, and MIP-2) upon *in vitro* culture in comparison with TNBS mice (Fig. 3C). These findings suggest that, in addition to the reduction in inflammatory infiltration, cortistatin administration deactivates the inflammatory response in the colonic mucosa. The broad antiinflammatory activity of cortistatin in the colon was accompanied by down-regulation of the systemic inflammatory response implicated in colonic inflammation (Fig. 3D). Cortistatin decreased the TNBS-induced serum levels of the proinflammatory cytokines TNF-α, IL-1β, IL-6, and MIP-2, and of serum amyloid A (SAA), a hepatic acute phase protein involved in tissue damage in inflammatory conditions.
histopathologic severity of the wasting disease, abrogating body weight loss, diarrhea, and intestinal inflammation and reduced the high mortality caused by this syndrome. From a therapeutic point of view, it is extremely important to take into account the ability of delayed administration of cortistatin to ameliorate ongoing disease and that an initial treatment with cortistatin prevented recurrence of the disease given a second dose of TNBS, which fulfills an essential prerequisite for an anticolitic agent, because treatment is started after the onset of CD patients.

There are several potential mechanisms by which cortistatin therapy can modulate the effector phase of TNBS colitis. CD and TNBS-induced colitis are characterized by transmural inflammation of the colon because of an IL-12-driven, Th1 cell-mediated response to TNBS-haptenated colonic proteins and/or crossreactive luminal antigens (4). During the effector phase of bowel inflammation, both innate and acquired immune responses overlap, and multiple inflammatory mediators are involved. Cortistatin strongly reduced mucosal inflammation by down-regulating the production of a wide panel of mediators involved in the local and systemic inflammatory response. Chemokines are responsible for the mucosal infiltration and activation of various leukocyte populations, which contribute to colitis development (15). The fact that cortistatin treatment reduced the production of a plethora of chemokines could partially explain the absence of inflammatory infiltrates in the colonic mucosa of cortistatin-treated mice, being especially relevant for chemokines, such as MIP-2 (chemotactic for neutrophils), IP-10 (for Th1 cells), and Rantes/MIP-1α (for macrophages and T cells), all of which are involved in CD pathogenesis (15). In addition to the regulation of cell recruitment to the lamina propria during colitis, cortistatin regulates the inflammatory cell activation and cytokine production. Thus, cortistatin downregulated the production of the proinflammatory cytokines TNF-α, IFN-γ, IL-6, IL-1β, IL-12, IL-15, IL-17, IL-18, and macrophage migration inhibitory factor by mucosal immune cells and increased the levels of the antiinflammatory cytokine IL-10. The decrease in inflammatory mediators could be the consequence of a diminished infiltration of inflammatory cells in the colonic mucosa in the cortistatin-treated TNBS mice. However, the fact that LPMC isolated from cortistatin-treated mice produced lower levels of proinflammatory cytokines upon in vitro activation argues against this hypothesis, which suggests that, in addition to the reduction in inflammatory infiltration, cortistatin administration deactivates the inflammatory response. In this study, we also demonstrated that cortistatin acts as a macrophage-deactivating factor by down-regulating the production of a wide board of inflammatory mediators. Therefore, it is plausible that deactivation of resident and infiltrating mucosal macrophages is a major mechanism involved in the antiinflammatory action of cortistatin in IBD. The local antiinflammatory action of cortistatin was also evident systemically. Of special consideration is the cortistatin reduction of SAA, an acute phase protein used for clinical monitoring of CD (16), which has been associated with tissue damage in several inflammatory conditions (17), although its precise role in intestinal inflammation is not known.

TNBS-induced colitis also is a Th1-mediated disease, requiring T cell activation as the central initiating event that subsequently leads
to macrophage recruitment and activation (4). The bias toward Th1 cytokines (mainly IFN-γ and TNF-α) is crucial in the establishment of chronic inflammation. Our results demonstrate that the expression of the Th1-type cytokines IFN-γ and TNF-α in the colon is down-regulated in response to cortistatin after the induction of TNBS colitis. It appears that the inhibition of the Th1 response is caused by a direct action on LPMC and draining MLN cells, because LPMC and MLN cells obtained from cortistatin-treated animals are refractory to Th1 cell stimulation. In contrast to IFN-γ and TNF-α, cortistatin increased the production of IL-10 in vivo. The effect of cortistatin on IFN-γ and IL-10 production by T cells seems to be independent of the action of the peptide on macrophages, because cortistatin directly affects T cells in the absence of antigen-presenting cells (our unpublished data). However, the fact that cortistatin increased IL-10 but not IL-4 production in CD4 T cells from LPMC and MLN argues against a shift toward Th2 responses. IL-10 has been recently recognized as a signature cytokine for a subset of CD4 T cells that exert regulatory functions (18). Active suppression by IL-10-secreting regulatory T cells (Treg) plays a key role in the control of self-antigen-reactive T cells and the induction of peripheral tolerance in vivo (18). In fact, deletion or dysfunction of these suppressive cells result in the appearance of autoimmune inflammatory disorders, especially in the intestine (19–21). Our data suggest that cortistatin induces the generation/activation of IL-10-secreting Treg cells. This finding correlates with the fact that cortistatin increased the colonic expression of CCR-4 and CCR-8, two chemokine receptors expressed in Treg cells (22, 23). Cortistatin may favor the recruitment of Treg to the inflamed mucosa. However, we did not observe a significant increase in the expression of ligands for CCR4 and CCR8 (CCL17 and CCL1) in the colon after cortistatin treatment. Although the mechanism is still unknown, preliminary experiments indicate that cortistatin induces the differentiation of tolerogenic dendritic cells with capacity to generate IL-10-secreting Treg cells (our unpublished results).

The improvement in wasting disease seen in mice treated with cortistatin (~90%) compares favorably to that achieved with other therapies, such as blocking of IL-12, TNF-α, IL-6 or IFN-γ, or treatment with prednisolone or mesalamine (4, 13, 24–27), some of them widely used in treating IBD patients. The capacity of cortistatin to regulate a wide spectrum of inflammatory mediators, in addition to suppression of Th1-type responses and potential generation of Treg cells, might offer a therapeutic advantage over neutralizing Abs directed against a single mediator.

Cortistatin shares many structural and functional properties with somatostatin. The amino acid sequences of these cyclic peptides, the gene structures, the partial coexpression, and the activation of common receptors and signaling pathways suggest that the two peptides exert similar functions. Although somatostatin-deficient mice do not display an overt phenotype (28), the lack of increased cortistatin expression in these mice argues against a compensatory role of cortistatin. The distinct functions of cortistatin in the nervous system (5, 6) and the present work support this hypothesis. In fact, cortistatin is significantly more efficient in preventing colitis development than somatostatin or its agonist octreotide. The superior potency of cortistatin in reducing inflammation may reside in its capacity to activate different receptors and transduction pathways. Whereas somatostatin and octreotide only bind to somatostatin receptors, cortistatin also can activate other receptors, including the receptor of ghrelin, a orexigenic hormone recently identified as a potent antiinflammatory factor (10–12). We have found that ghrelin also protects against TNBS-induced colitis by inhibiting the production of inflammatory cytokines and chemokines by macrophages by down-regulating the production of Th1 cytokines by CD4 T cells and by increasing the production of IL-10 by macrophages and T lymphocytes (29). Therefore, the possibility exists that cortistatin is exerting its therapeutic effect on IBD, at least partially, through the ghrelin receptors. Indeed, cortistatin effects on inflammation and colitis were partially reversed by somatostatin- and ghrelin-receptor antagonists. Finally, the participation of cortistatin-specific receptors, not yet identified, cannot be ruled out.

Of physiological relevance is the observation that the expression of cortistatin is increased in activated inflammatory cells (7, 8). Although the levels of cortistatin have not been yet measured in CD patients, it is attractive to speculate that the body responds to an exacerbated inflammatory response by increasing the peripheral production of endogenous antiinflammatory factors, including cortistatin. In our study, the animals did not exhibit side effects, probably because a short period of treatment with the peptide is enough to get a significant disease remission without recurrence, and plasma cortistatin levels only slightly increased after cortistatin treatment (32 versus 62 pg/ml). Extending the use of cortistatin to the human system, however, will depend on the peptide dosage and the expression of somatostatin/cortistatin/ghrelin receptors in human immune cells, because species-related differences in expression have been found (7–9). In addition, based in its somatostatin-like structure, cortistatin should be unstable and susceptible to
degradation, making the peptide not very useful for clinical application in theory. However, the in vivo effects showed by cortistatin in this model are very convincing. Therefore, some of the fragment resultants of the degradation of the peptide could still retain biological functions, as previously described in other systems (30). In fact, no increased levels of cortistatin were detected in colons of cortistatin-treated mice.

In summary, this work identifies cortistatin as an immunomodulator factor with the capacity to deactivate the inflammatory response in vivo at multiple levels and provides a powerful rationale for the assessment of the efficacy of cortistatin as a therapeutic approach to the treatment of CD.

Methods

Induction of Colitis and Study Design. Colitis was induced in 6- to 8-week-old BALB/c and SJL mice (The Jackson Laboratory) as described in ref. 13. Briefly, 3 mg of TNBS (Sigma) in 50% ethanol (to break the intestinal epithelial barrier) was administered into the colon via a catheter. Control mice received 50% ethanol alone. Animals were treated i.p. with medium or with different concentrations: 0.05–2.0 mg per mouse; 6–250 μg/kg of cortistatin 1–29 (American Peptide, Sunnyvale, CA), somatostatin, or octreotide. To study the therapeutic effect of the delayed administration of cortistatin on established colitis, cortistatin or octreotide. To study the therapeutic effect of the delayed administration of cortistatin on established colitis, cortistatin (2 nmol per mouse) was injected i.p. for 3 consecutive days starting 6 days after TNBS instillation. In some experiments, cyclo-

somatostatin or [α-Lys-3]-growth hormone releasing peptide 6 (Lys-GHRP-6; Sigma) was injected i.p. (250 μg per mouse) with cortistatin or octreotide. To study the therapeutic effect of the delayed administration of cortistatin on established colitis, cortistatin (2 nmol per mouse) was injected i.p. for 3 consecutive days starting 6 days after TNBS administration. To study the effect on disease recurrence, 1.5 mg of TNBS was administered at days 0 and 9, and cortistatin (2 nmol per mouse) was injected i.p. 12 h after the first TNBS infusion. Animals were monitored daily for appearance of diarrhea, loss of body weight, and survival. Some animals were killed at the peak of the disease (day 3), blood samples were collected by cardiac puncture, and a segment of the colon was excised for macroscopic damage evaluation and weighed. Tissue collection by cardiac puncture, and a segment of the colon was collected for histopathological analysis, sections were stained with hematoxylin and eosin, and inflammation was graded from 0 to 4 as follows in a blinded fashion: 0, no signs of inflammation; 1, low leukocyte infiltration; 2, moderate leukocyte infiltration; 3, high leukocyte infiltration, moderate fibrosis, high vascular density, thickening of the colon wall, moderate goblet cell loss, and focal loss of crypts; 4, transmural infiltrations, massive loss of goblet cell, extensive fibrosis, and diffuse loss of crypts. For immunohistological analysis, sections were stained with 5 μg/ml FITC- and phycoerythrin-labeled anti-CD4, anti-CD11b, or anti-TNF-α mAbs and examined by fluorescent microscopy.

Macroraph Cultures. Peritoneal exudate cells obtained from BALB/c mice were incubated in complete medium at 106 cells per milliliter, and, after 2 h at 37°C, nonadherent cells were removed. Macrophage monolayers were incubated with complete medium in the absence (unstimulated) or presence of 1 μg/ml LPS from Escherichia coli serotype 055:B5 (Sigma) and different concentrations of cortistatin. Supernatants were collected at different times, and cytokine levels were determined. The amount of NO formed was estimated from the accumulation of the stable NO metabolite nitrite by the Griess assay.

Data Analysis. All values are expressed as means ± SD. The differences between groups were analyzed by Mann–Whitney U test and, if appropriate, by Kruskal–Wallis ANOVA test. Survival curves were analyzed by the Kaplan–Meier log-rank test. Changes in body weight were compared by use of the Wilcoxon matched-pair signed-rank test.

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