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The Immunomodulatory Action of Sialostatin L on Dendritic Cells Reveals Its Potential to Interfere with Autoimmunity

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Sialostatin L (SialoL) is a secreted cysteine protease inhibitor identified in the salivary glands of the Lyme disease vector Ixodes scapularis. In this study, we reveal the mechanisms of SialoL immunomodulatory actions on the vertebrate host. LPS-induced maturation of dendritic cells from C57BL/6 mice was significantly reduced in the presence of SialoL. Although OVA degradation was not affected by the presence of SialoL in dendritic cell cultures, cathepsin S activity was partially inhibited, leading to an accumulation of a 10-kDa invariant chain intermediate in these cells. As a consequence, in vitro Ag-specific CD4+ T cell proliferation was inhibited in a time-dependent manner by SialoL, and further studies engaging cathepsin S−/− or cathepsin L−/− dendritic cells confirmed that the immunomodulatory actions of SialoL are mediated by inhibition of cathepsin S. Moreover, mice treated with SialoL displayed decreased early T cell expansion and recall response upon antigenic stimulation. Finally, SialoL administration during the immunization phase of experimental autoimmune encephalomyelitis in mice significantly prevented disease symptoms, which was associated with impaired IFN-γ and IL-17 production and specific T cell proliferation. These results illuminate the dual mechanism by which a human disease vector protein modulates vertebrate host immunity and reveals its potential in prevention of an autoimmune disease. The Journal of Immunology, 2009, 182: 7422–7429.

Hard ticks feed on their hosts for an extended period of time, sometimes exceeding 10 days, over which a full range of inflammatory and immune reactions take place at the feeding site as well as systemically on the host body. Thus, active modulation of the host immune response by tick saliva is required for the completion of its long-lasting blood feeding, which facilitates the transmission of pathogens (1). Indeed, apart from the Lyme disease agent Borrelia burgdorferi, ticks efficiently transmit protozoa and viruses that cause a variety of diseases in humans (2). According to the Centers for Diseases Control and Prevention (Atlanta, GA), Lyme disease is the most common tick-borne disease in North America and one of the fastest growing infectious diseases in the United States (3).

A key effect of tick saliva on vertebrate immunity is the marked suppression of T cell proliferation accompanied by down-regulation of Th1 cytokines such as IFN-γ and IL-2 (4–6). Reduced proliferative activity of Con A on T lymphocytes was found in different hosts upon infestation with Rhipicephalus sanguineus, Ixodes ricinus, and Dermacentor andersoni (7–9). In vitro mitogen-driven and Ag-specific proliferation has also been demonstrated to be inhibited by tick saliva from different species (10–13). Although the immunomodulatory properties of Ixodes salivary “cocktail” were revealed more than two decades ago (13), the constituents that account for these activities have not been fully characterized. Certain molecular components common to ticks, such as PGES, have been shown to modulate lymphocyte proliferation (13, 14). Moreover, some proteins have been associated with such suppressive activity, including a 36-kDa protein from D. andersoni (15), an immunosuppressor from I. ricinus (16), an IL-2 binding protein (17), and Salp15 from I. scapularis (18, 19). We have recently characterized a secreted cysteine protease inhibitor from I. scapularis salivary glands that selectively targets a limited subset of human cathepsins (20, 21). This inhibitor displays high affinity for cathepsin L ($K_d \sim 10^{-10} M$), prompting us to name it sialostatin L (SialoL). When the sialostatin gene was silenced using RNA interference in ticks, the vertebrate host (in that case, rabbits) recognized ticks more quickly, leading to tick feeding impairment (21). Taking into consideration both the pivotal role of sialostatin’s enzymatic targets in Ag processing/presentation (22, 23) and the faster immune recognition of ticks in the absence of sialostatin secretion from their salivary glands (21), we have undertaken an investigation into the mechanism of action of this protein. More

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3 Abbreviations used in this paper: SialoL, sialostatin L; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; Ii, invariant chain; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; WT, wild type.
specifically, we demonstrate that SialoL inhibits microbial-induced maturation of dendritic cells (DCs) as well as Ag-specific T cell proliferation. Furthermore, we show that cathepsin S inhibition accounts for the observed SialoL-mediated effects on immunity and that in vivo treatment of mice with SialoL impairs early CD4+ T cell expansion upon antigenic stimulation and in vitro recall responses. Finally, using a murine model for multiple sclerosis, we show that in vivo administration of SialoL delays disease onset and prevents its symptoms. Collectively, these data shed light on the immunomodulatory mechanism of SialoL and its preventive potential against an autoimmune disease. Beyond the basic knowledge of the mechanisms that ticks have developed to successfully obtain a blood meal, the current work shows SialoL to be an attractive candidate in the development of novel drug formulations for the treatment of immunity-related pathological conditions such as autoimmune diseases.

Materials and Methods

Unless otherwise indicated, protocols followed standard procedures (24) and experiments were performed at room temperature (25 ± 1°C). All water used was of 18-MΩ quality produced by a MilliQ apparatus (Millipore). If not otherwise stated, all reagents were purchased from Sigma-Aldrich and all cells were cultured at 37°C under an atmosphere of 5% CO2. All experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of the National Institutes of Health.

SialoL preparation and LPS decontamination

The SialoL gene was overexpressed in Escherichia coli, and the corresponding active protein was purified in 0.8 mM stock solution as previously described (20, 21). Any potential LPS contamination in the stock solution was removed by Arvys Proteins using detergent extraction; endotoxin presence by the end of the procedure was estimated as <4 × 10−5 endotoxin U/μg protein (roughly, <3 × 10−14 g of endotoxin per microgram of protein) with a sensitive fluorescent-based endotoxin assay (PyroGene recombinant factor C endotoxin detection system; Lonza Biologies).

Mice and cell populations

Female mice (6–10 wk old) were used. C57BL/6 and BALB/c were purchased from Charles River Laboratories. B6.PL-Thy1a/CyJ mice were purchased from The Jackson Laboratory. OT-II mice (which express TCR specific for OVA peptide 323–339) were purchased from Taconic Farms. Cathepsin S+/− (C57BL/6 background) and L−/− (C57BL/6/S129 background) mice and their wild-type (WT) littermates were bred at the Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School (Boston, MA). Animals were maintained at an American Association of Laboratory Animal Care-accredited facility at the National Institutes of Health (Bethesda, MD).

Bone marrow-derived DCs from cathepsin S−/− and L−/− mice and their respective WT littermates were generated as previously described (14). After 6 days of culture with GM-CSF, nonadherent cells were collected and enriched for CD11c− cells as described elsewhere (25). CD4− T cells were purified from spleens of OT-II mice using a Dynal mouse CD4-negative isolation kit (Invitrogen).

LPS-induced maturation of DCs

DCs from C57BL/6 mice were cultured at 106 cells/well in flat-bottom 96-well cluster plates (Costar; Corning) and preincubated for 2 h with medium in the presence or absence of SialoL. Subsequently, they were stimulated with 50 ng/ml ultrapure LPS (InvivoGen). Cell-free supernatants were collected for 72 h in the presence or absence of SialoL. They were then washed three times in cold PBS and levels of these cytokines were measured using OptEIA ELISA sets (Bender Diagnostics).

Western blot analysis

DCs were prepared as described above and incubated with medium or 3 μM SialoL, for 24 h. They were then washed three times in cold PBS and cell pellets were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 0.5% Nonidet P-40, and 5 mM MgCl2) on ice for 2 h. After centrifugation at 15,000 × g for 10 min, volumes of samples each containing the same amount of protein were dissolved into nonreduced lithium dodecyl sulfate sample buffer (Invitrogen) and either boiled or nonboiled samples were separated by electrophoresis on a 12% SDS-polyacrylamide gel. Separated proteins were transferred onto nitrocellulose filters that were then probed with anti-invariant chain (li) (CD74; BD Pharmingen) and anti-cathepsin S (Santa Cruz Biotechnology). HRP- or alkaline phosphatase-conjugated secondary Abs were used for signal detection. Filters were developed with Western Blue stabilized substrate for alkaline phosphatase (Promega) or SuperSignal West Pico chemiluminescent substrate for HRP (Thermo Fisher Scientific).

OVA degradation in DCs

DCs were prepared as described above and preincubated with medium or 3 μM SialoL for 3 h. DQ-OVA (1 μg/ml; Molecular Probes) was then added to the cultures and further incubated for 2 h. A control group consisted of cells incubated with medium but not with DQ-OVA. Cells were removed and washed with PBS with 1% FBS at 4°C, and OVA degradation inside the cells was analyzed by flow cytometry (FL-1 fluorescence channel).

Ag-specific CD4+ T cell proliferation and MLR

DCs (2.5 × 105/well) were preincubated at different time points with medium or 3 μM SialoL, as indicated. Then, CD4+ T cells (105/well) were added and cultures were incubated for 72 h in the presence or absence of 1 μg/ml OVA (Inject OVA; Pierce).
For MLR, total splenocytes from BALB/c mice (2.5 × 10^6/well) were preincubated for 3 h with medium in the presence or absence of SialoL. Subsequently, splenocytes from OT-II mice (2.5 × 10^6/well) were added and incubated for 72 h. Proliferation was assessed by adding 10% (v/v) alamarBlue (TREK Diagnostic Systems) in the last 48 h of incubation. Absorbance was measured at 570 and 600 nm as previously described (26).

**Adoptive transfer and cell proliferation in vivo and in vitro**

CD4^+ T cells from OT-II mice (Thy1.2) were purified as described above and labeled with 2 μM CFSE (Invitrogen) according to the manufacturer’s instructions. B6.PL-Thyla/CyJ mice (Thy1.1) were i.v. injected with 2.5 × 10^6 CD4^+ T cells and, 1 day later, immunized with 10 μg of OVA emulsified in CFA. A volume of 100 μl of emulsion per site was s.c. injected into two sites on the flanks of mice near the tail. Six hours before immunization, concomitant with the immunization, and 6 h after the immunization, mice received s.c. injections of BSA or SialoL (10 μg/injection) into the same sites of the flanks. Additional control groups consisting of mice receiving only CFA in PBS (as a negative control for the immunization) and PBS as a treatment were analyzed. Lymph nodes (LN) were collected from mice receiving the immunization or control groups 4 days after immunization and cells were stained with fluorochrome-labeled Abs specific for CD11c, CD80, CD86, and MHC class II (I/II) and analyzed by flow cytometry. The data shown were gated from live CD11c^+ cells.

**Induction of experimental autoimmune encephalomyelitis (EAE)**

Induction of EAE was performed according to a previously published protocol (27). Female C57BL/6 mice were immunized with 200 μg of myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide (MOG p35–55) (Anaspec) emulsified in IFA together with 5 mg/ml Mycobacterium tuberculosis H37RA. A volume of 100 μl of emulsion per site was s.c. injected into two sites on the flanks of mice near the tail. At days 0 and 2 following the initial injections, animals received additional i.p. injections of 200 ng of pertussis toxin. Six hours before immunization, concomitant with the immunization, and 6 h after the immunization, mice received s.c. injections of PBS, SialoL, or SialoL2 (10 μg/injection) on the flanks into the same sites as those that received the immunization. Mice were scored daily for clinical assessment of disease based on the following criteria: 0, normal; 1, limp tail or hind limb weakness; 2, limp tail and hind limb weakness; 3, paralysis of both hind limbs; 4, paralysis of both hind limbs and forelimb; 5, paralysis and moribund or dead. Food and water was made accessible to immobile animals, and moribund animals with a score of 6 were euthanized.

**Cell proliferation and cytokine production by LN cells**

The left and right inguinal LNs from naive or MOG-immunized mice treated with BSA (negative control) or SialoL were removed 10 days after immunization. At this period, mice do not show any clinical symptoms of EAE. The cells were dispersed through a 40 μM cell strainer (BD Falcon), and RBCs were hypotonically lysed. The cells were washed twice and cultured at 2 × 10^6/well for 72 h in 96-well microplates (BD Falcon) with either medium or MOG (1 and 5 μg/ml). Proliferation was assessed by adding alamarBlue as described above, and levels of IFN-γ (BD Biosciences) and IL-17 (R&D Systems) were determined in cell-free supernatants.

**Statistical analysis**

Each experiment was performed at least three times and data are shown as mean ± SEM. Statistical differences were analyzed by Student’s *t* test and ANOVA. A value of *p* < 0.05 was considered statistically significant.
In addition, it seems that under denaturing conditions the complex $S$ displays a distinct migration pattern compared with free enzyme. (38.8 and was consistently found in four different experiments incubated DCs. This result was confirmed by densitometry (Fig. 3 band from SialoL-incubated DCs when compared with medium-

Next, we asked whether the effects of SialoL were mediated by its interaction with intracellular cathepsin. DCs cultured with medium

Results

SialoL inhibits LPS-induced maturation of DCs

Given that DCs are a major resident cell type in the skin and that SialoL is present in I. scapularis salivary secretion (20), we first tested whether the DC response to microbial stimulation is affected by the presence of SialoL. LPS-induced production of IL-12 by DCs was inhibited in a concentration-dependent manner by SialoL, reaching ~60% inhibition at 3 $\mu$M (Fig. 1A; $p < 0.05$). TNF-$\alpha$ production was marginally inhibited in the same conditions, reaching ~25% inhibition at 3 $\mu$M SialoL (Fig. 1B; $p < 0.05$). None of the mentioned effects appear to be associated with alterations in IL-10 production, as no differences were observed in its production in the absence or presence of SialoL (Fig. 1C).

We next tested whether the expression of costimulatory molecules was affected by SialoL. Incubation with SialoL alone did not significantly change the expression of CD80 or CD86 in DCs when compared with control cells (incubated with medium only). However, preincubation of DCs with SialoL reduced the expression of these molecules by 40% of the level induced by LPS (data not shown).

SialoL binds cathepsin S inside DCs and affects Ii processing

Next, we asked whether the effects of SialoL were mediated by its interaction with intracellular cathepsin. DCs cultured with medium or SialoL were extensively washed, lysed, and blotted to detect cathepsin S. Fig. 3A shows a reduced intensity of the cathepsin S band from SialoL-incubated DCs when compared with medium-incubated DCs. This result was confirmed by densitometry (Fig. 3B) and was consistently found in four different experiments (38.8 ± 6.7% reduction; $p < 0.05$), suggesting the formation of a SDS-resistant intracellular complex where SialoL-bound cathepsin S displays a distinct migration pattern compared with free enzyme. In addition, it seems that under denaturing conditions the complex is preserved in nonboiled samples, preventing Ab recognition of the differentially migrating SialoL-bound cathepsin S. To exclude the possibility that SialoL affected the expression levels of cathepsin S, the samples were boiled to dissociate the complex and, in fact, the boiled samples presented bands of similar intensities, further confirming our hypothesis (Fig. 3, A and B). Similar results were found upon cell incubation with $N$-morpholinurea-leucine-homophenylalanine-4vinylsulfone-phenyl, also known as LHVS, a well-characterized cathepsin S inhibitor (data not shown) (28).

We next investigated the effects of cathepsin S inhibition on Ag degradation and Ii cleavage, two essential steps for Ag presentation (23). Regarding the Ag degradation, DCs were preincubated with medium or SialoL and pulsed with DQ-OVA, which emits fluorescence upon proteolysis. Fig. 3C shows that DQ-OVA fluorescence is equally detected in DCs in the presence or absence of SialoL, suggesting that SialoL does not affect this step, which is known to have the participation of additional cathepsins beyond cathepsin S (23). To evaluate Ii degradation, DCs were incubated with medium or SialoL, and after 24 h the Ii intermediates were detected in cell lysates using anti-Ii (CD74) Ab. Fig. 3D shows that the p31 and p41 isoforms of Ii (p31-Ii and p41-Ii) are equally detected in cell lysates from both groups whereas Ii-p10, whose cleavage is dependent on cathepsin S (29, 30), accumulates in the cells incubated with SialoL but not in control DCs. Additionally, SDS-stable MHC class II/II-p10 complexes can be dissociated...
upon boiling (29) and, in fact, boiled cell lysates from SialoL-incubated DCs presented a stronger Ii-p10 band (Fig. 3D). SialoL inhibits Ag-specific CD4+/H11001 T cell proliferation but not MLR in vitro

To determine whether the inhibition of DC maturation and Ii degradation correlate with a functional defect in Ag presentation, we next investigated the modulation of Ag-dependent T cell proliferation by SialoL. A time-dependent inhibition of OVA-specific CD4+/H11001 T cell proliferation was observed in the presence of SialoL, reaching ~65% upon 3 h of preincubation with the inhibitor (Fig. 4A; p < 0.05). In contrast, the MLR was not affected by preincubation with SialoL at any of the concentrations tested ranging from 0.1 to 3 μM, excluding SialoL-unspecific toxicity (Fig. 4B). Collectively, these data suggest that SialoL affects DC maturation and function rather than having a direct effect on T cells.

SialoL effect in Ag-specific CD4+/H11001 T cell proliferation is mediated by cathepsin S inhibition

To further demonstrate the cathepsin S-dependent inhibitory activity of SialoL on T cell proliferation, SialoL-exposed or -unexposed DCs from cathepsin L−/− and S−/− mice were used as APCs in the OVA-induced proliferation assay. Cathepsin L−/− DCs presented a similar proliferative stimulatory ability when compared with WT DCs (Fig. 5A). Upon preincubation with SialoL, both WT and cathepsin L−/− DC cultures produced a reduction in T cell proliferation (Fig. 5A; p < 0.05). In contrast, T cell proliferation in cathepsin S−/− DC cultures was strongly diminished when compared with WT DCs in the absence of SialoL (Fig. 5B; p < 0.05), whereas preincubation of these cultures with SialoL inhibited proliferation induced by WT and cathepsin S−/− DCs (Fig. 5B; p < 0.05). Because cathepsin S−/− DCs are not expected to stimulate OVA-specific T cell clones (29), the remaining proliferative activity presented by cathepsin S−/− DC cultures suggests a minor contamination with APCs from the donor (OT-II mice) in the CD4+/H11001 T cell preparation, as they were purified by negative selection. Consistent with this concept, we observed a complete lack of T cell proliferation in cathepsin S−/− DC cultures incubated with SialoL (Fig. 5B). Altogether, these results suggest that SialoL inhibits Ag-specific T cell proliferation by a cathepsin S-dependent mechanism.

In vivo treatment with SialoL during OVA immunization impairs early T cell expansion and late recall response of immune mice

We next evaluated whether the inhibitory effects of SialoL on T cell proliferation can be also achieved in vivo. To do so, recipient B6.PL-Thy1a/CyJ mice (Thy 1.1) received CFSE-labeled CD4+ T cell proliferation and recall response.

A–F, Adoptive transfer experiment. B6.PL-Thy1a/CyJ mice were i.v. injected with 2.5 × 10^6 CD4+ T cells from OT-II mice labeled with CFSE. One day later, mice were immunized at two sites of the flanks with 10 μg of OVA emulsified in CFA and treated with BSA or SialoL, as described. After 96 h, the LNs were removed and proliferation was measured by flow cytometry based on CFSE dilution of gated CD4+/Thy1.2+ cells. Nonimmunized mice (A and B), BSA-treated mice immunized with OVA (C and D), and SialoL-treated mice immunized with OVA (E and F) are shown. G, Recall response. C57BL/6 naïve mice were immunized at two sites on the flanks with 10 μg of OVA emulsified in CFA and treated with BSA or SialoL, as described. Thirty days later, LN cells were stimulated in vitro with increasing concentrations of OVA or Con A for 72 h and proliferation was measured. *, p < 0.05 vs OVA + BSA group.
T cells from OT-II mice (Thy 1.2) and were subsequently immunized with OVA in the presence of BSA (control protein) or SialoL (see Materials and Methods for immunization protocol). CFSE dilution was analyzed 4 days after immunization. As expected, transferred cells represented only a small fraction of total cells in a nonimmunized recipient (Fig. 6A) and did not proliferate (Fig. 6B). In OVA-immunized mice, transferred CD4+ T cells represented 3% of total cells (Fig. 6C) and virtually all of them proliferated, presenting up to 8–9 divisions (Fig. 6D). Nevertheless, in SialoL-treated mice immunized with OVA, transferred CD4+ T cells represented <1% of total cells (Fig. 6E), and nearly half of them did not proliferate (no CFSE dilution; Fig. 6F).

In another set of experiments, naive C57BL/6 mice were treated with SialoL or BSA and subsequently immunized with OVA. After 30 days, their LN cells were isolated and restimulated in vitro and their proliferative response was compared. Proliferation of LN cells from SialoL-treated animals was reduced in comparison with that in BSA-treated animals, and this inhibition was consistent in all OVA concentrations used upon in vitro restimulation (Fig. 6G; p < 0.05). However, the polyclonal proliferation induced by Con A was not affected in any group (Fig. 6G), suggesting that SialoL treatment affects Ag-specific proliferation but not mitogen-driven proliferation. Of note, when a much higher amount of OVA (10 times more) was used during immunization, the inhibitory effect on the proliferative response was barely or not observed (data not shown), indicating that an excessive amount of Ag is capable of surpassing this inhibitory activity.

Prevention of EAE by SialoL

We next determined whether the observed in vivo effects of SialoL could be extended to an autoimmune disease setting. We used the EAE model because this disease is caused by autoreactive T cells primed by DCs after immunization of mice with Ags derived from a myelin sheath protein (31). Therefore, the disease onset depends on the functionality of DCs. During MOG immunization, mice were treated with PBS, SialoL, or SialoL2; the latter being a second cystatin from I. scapularis with at least 500 times lower affinity for cathepsin S (21). Strikingly, SialoL prevented disease symptoms at the onset of EAE between days 12 and 15 after immunization (Fig. 7A). In contrast, mice receiving SialoL2 displayed no differences in clinical score compared with the PBS-injected group, serving as an additional negative control group (Fig. 7A). After the peak of EAE onset, in the late milder phase of the disease (day 16 to the end of the monitoring period), there was no difference between the experimental groups. In addition, because SialoL was previously shown to inhibit neutrophil migration (20), we tested whether this anti-inflammatory activity affects the disease when T cells are already primed by the Ag. Treatment of animals with SialoL at days 12, 14, and 16 after MOG immunization had no effect on EAE development (data not shown).

The inguinal LNs that drain the MOG injection site were dissected at day 10 following MOG immunization (before the onset of the clinical signs of EAE). We then evaluated the proliferative response and cytokine production of LN cells upon in vitro restimulation with
MOG. In animals treated with BSA at the time of MOG immunization the LN cells were strongly responsive to MOG restimulation, proliferating and producing IFN-γ and IL-17 (Fig. 7, B–D). In contrast, LN cells from mice treated with SialoL at the time of MOG immunization presented a weak proliferative response and barely detectable cytokine production upon in vitro MOG restimulation (Fig. 7, B–D). This decreased response was not associated with increased IL-10 or TGF-β production (data not shown).

Discussion

We have previously shown that SialoL is a constituent of the I. scapularis salivary secretion (20) and is therefore coadministered with the pathogens transmitted when these ticks are attached to the skin. In this environment, DCs are a major resident cell type and are probably among the first cells activated by pathogen products such as TLR ligands from these microorganisms. Thus, we determined whether the DC response to LPS, a typical TLR ligand, is affected in the presence of SialoL, by measuring the cytokines and costimulatory molecules involved in the maturation process of these cells. In vitro LPS-induced production of IL-12 (Fig. 1A) and, less extensively, TNF-α (Fig. 1B) was affected by the presence of SialoL in the culture. This observed down-modulation is apparently not associated with changes in IL-10 production, as levels of this cytokine were similar in the presence or absence of SialoL (Fig. 1C). We have previously demonstrated that I. scapularis saliva inhibits LPS-induced TNF-α and IL-12 production and increases IL-10 production by DCs and that PGE₂ is the major low m.w. mediator responsible for this activity in saliva (14). The results presented here suggest that inhibition of TNF-α and IL-12, but not the increase of IL-10 production, may also be attributed to salivary SialoL, which is not present in the low m.w. fraction characterized in our previous work. Additionally, CD80 and CD86 expressions in DCs, which are increased upon incubation with LPS, are reduced in the presence of SialoL (Fig. 2). Nevertheless, the participation of cytokine proteases in DC maturation has never been reported before is currently under investigation in our laboratory.

DCs are also potent APCs that initiate adaptive immune responses through the activation of T cells. Ag processing and presentation by APCs is a complex process with two concomitant pathways, both dependent on cathepsins (23, 32). Ag degradation, which produces antigenic peptides to bind MHC class II molecules, is a process only partially dependent on cathepsin S, and other cathepsins are able to replace it in the absence of the former (23). In contrast, the final proteolysis of the ιι associated with MHC class II molecules is exclusively dependent on cathepsin S (23, 34). Western blot analysis consistently showed a partial decrease of cathepsin S intensity in the cell lysates of DCs incubated with SialoL that suggests an intracellular complex formation between SialoL and cathepsin S, a finding that is consistent with picomolar affinity between the enzyme and the inhibitor (21). This binding was reversible upon boiling (Fig. 3, A and B), indicating that binding of a tight inhibitor to cathepsin S either masks or distorts the epitope recognized by the Ab used to reveal this enzyme in Western blot assays, and experiments performed under the same conditions with the well-characterized cathepsin S inhibitor LHVS (28, 30) confirmed these findings (data not shown). To investigate whether cathepsin S inhibition by SialoL interfered with the two essential steps for Ag presentation, we firstly analyzed DQ-OVA cleavage inside DCs. Fig. 3C shows that DQ-OVA fluorescence is detected equally in DCs in the presence or absence of SialoL, suggesting that the presence of SialoL inside these cells is not associated with a general lysosomal poisoning. Then, the iip10 intermediate, whose cleavage is catalyzed by cathepsin S, was detected in cell lysates. Strikingly, iip10 was detected in cells incubated with SialoL (Fig. 3D), and this accumulation is indicative of a defective peptide loading into MHC class II molecules (29). The inhibitory role of SialoL in OVA-specific proliferation (Fig. 4A) confirms the biological significance of our previous findings. This result is consistent with previous studies conducted with nonspecific and specific inhibitors of cysteine proteases (34, 35). However, the strict and narrow target specificity of SialoL (20) restricts its action to Ag-specific proliferation systems, as no inhibitory activity on MLR has been observed (Fig. 4B). In addition, the defective T cell proliferation seen when using cathepsin S⁻/⁻ DCs, but not cathepsin L⁻/⁻ DCs (Fig. 5), is in agreement with a previous work (23). Thus, our results confirm a dominant role for the inhibition of cathepsin S by SialoL over that of cathepsin L as the molecular mechanism that accounts for its effects on Ag presentation. Together with the above-described activity on DC maturation (Figs. 1 and 2), these data suggest that SialoL does not directly affect T cell function but rather interferes with both TLR-dependent and Ag-dependent functions of DCs.

The immunomodulatory action of SialoL was also observed in vivo. More specifically, SialoL treatment reduced CD4⁺ T cell proliferation in vivo relative to BSA treatment upon OVA immunization (Fig. 6), suggesting that APCs exposed to the inhibitor displayed reduced functionality. Inhibition of early T cell expansion by SialoL affected subsequent recall responses, as ex vivo recall response to OVA was impaired in mice receiving SialoL whereas Con A-induced proliferation was not affected (Fig. 6G). This suggests that the inhibitory action of SialoL in vivo is specific for Ag-dependent proliferation and not for mitogen-driven polyclonal proliferation. As a proof of principle, we tested SialoL activity in an animal model for human multiple sclerosis, i.e., EAE. The disease is mediated by autoreactive T cells that recognize Ags of the myelin sheath of the CNS, including myelin basic protein and MOG (36). Induction of EAE requires T cell responses initiated by DC priming of naive CD4⁺ T cells (31) and can also occur by passive transfer of myelin Ag-specific Th17 or Th1, but not Th2 cells (37–39). Markedly, treatment of mice with SialoL during the immunization phase of EAE concomitant with MOG injection resulted in delayed EAE development and milder disease symptoms in comparison with the PBS-treated group (Fig. 7A). SialoL2, a second cystatin described in I. scapularis saliva that displays similar inhibitory activity for cathepsin L but is a >500-fold weaker cathepsin S inhibitor in comparison to SialoL (21), presented no effect on development of the symptoms (Fig. 7A). In addition, draining LN cells from SialoL-treated mice were markedly less responsive to in vitro MOG restimulation than LN cells from control mice (Fig. 7, B–D). Based on the in vitro and in vivo results, it is reasonable to conclude that SialoL negatively affected DC maturation and function, thus interfering with MOG-specific T cell stimulation and causing the observed effect on disease onset. This scenario is further corroborated by the strongly inhibited proliferation of LN cells from SialoL-treated mice upon MOG restimulation in vitro. Additionally, cytokines involved in the development of EAE, namely IFN-γ and IL-17 (39–42), were suppressed, indicating that a defect in the priming of CD4⁺ T cells was the mechanism by which SialoL treatment suppressed EAE development. To our knowledge, this is the first work showing the preventive role of an ectoparasite protein on an autoimmune disease. This is in line with previous studies relating cathepsin S to autoimmune pathologies (43–45), a novel field that some pharmaceutical companies are beginning to explore with cathepsin S inhibitors in clinical trials (46). Thus, we propose that the SialoL/ cathepsin S axis could be used in designing novel treatments for autoimmune diseases.
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Disclosures
The authors have no financial conflict of interest.

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