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LF 15-0195 Inhibits the Development of Rat Central Nervous System Autoimmunity by Inducing Long-Lasting Tolerance in Autoreactive CD4 T Cells

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Experimental autoimmune encephalomyelitis (EAE) is a T cell-dependent autoimmune disease induced in susceptible animals by a single immunization with myelin basic protein (MBP). LF 15-0195 is a novel immunosuppressor that has been shown to have a potent immunosuppressive effect in several pathological manifestations. The purpose of this study was to investigate the effect of this drug on the induction and progression of established rat EAE and to dissect the mechanisms involved. We show that LF 15-0195 administration at the time of MBP immunization reduces the incidence and severity of EAE in Lewis rats. This drug also inhibits ongoing and passively induced EAE, indicating that LF 15-0195 affects already differentiated pathogenic lymphocytes. Compared with lymph node cells from untreated rats, lymphocytes from MBP-immunized rats treated with LF 15-0195 proliferated equally well in response to MBP in vitro, while their ability to produce effector cytokines and to transfer EAE into syngeneic recipients was significantly reduced. This phenomenon is stable and long-lasting. Indeed, neither IL-12 nor repeated stimulation with naive APC and MBP in vivo rendered MBP-specific CD4 T cells from protected rats encephalitogenic. In conclusion, LF 15-0195 treatment suppresses EAE by interfering with both the differentiation and effector functions of autoantigen-specific CD4 T cells. The Journal of Immunology, 2003, 170: 2179–2185.

Autoimmune diseases result from dysregulation of the immune system that targets its own organs, tissues, and cells for destruction. These diseases include >70 chronic disorders that collectively affect millions of individuals worldwide. In general, these diseases are associated with humoral or cell-mediated immune reactions against one or more of the body’s own constituents. Based on exciting results in transplantation, a number of conventional immunosuppressants, i.e., glucocorticoid, azathioprine, and cyclosporin A, are also clinically used for treating autoimmune diseases. Despite the widespread use of these immunosuppressive drugs, a firm conclusion about efficacy has been hard to come by. Furthermore, the long term administration of these drugs induces several side effects. Therefore, there is a need for new, powerful, and less toxic immunosuppressive drugs, which could lead to a more specific immunosuppression.

Among the different compounds recently developed, 15-deoxy sperguasin (DSG), a synthetic analog of sperguasin that was isolated as an antitumor compound from Bacillus laterosporus, appeared attractive. DSG has powerful immunosuppressive properties and differs from other immunosuppressive agents, both structurally and mechanistically (1–4). DSG is frequently more effective than popular immunosuppressants such as cyclosporin A, FK 506, or rapamycin at inducing immunosuppression (1, 5, 6). DSG can prolong the survival of, even induce tolerance to, tissue grafts in several animal models involving either allo- or xeno-disparities (7–9). DSG has also been effective in decreasing the severity of autoimmune diseases (10). However, DSG suffers from several drawbacks, such as its low chemical stability in aqueous solution and its susceptibility to oxidative metabolism in vivo, which limit its clinical use, particularly in chronic autoimmune diseases that may require long term administration. LF 15-0195, the recently developed analog of DSG devoid of the above-mentioned drawbacks, has improved immunosuppressive activity compared with DSG (11). LF 15-0195, which is now being investigated as a potential therapeutic drug for human inflammatory diseases, has demonstrated its efficacy in allotransplantation (12) and in Ab-mediated autoimmune diseases (13, 14). However, its effect on cell-mediated autoimmunity has not yet been tested.

In the present study we evaluate the effect of LF 15-0195 treatment on the development of rat experimental autoimmune encephalomyelitis (EAE). This inflammatory autoimmune disease of the CNS is considered an experimental model for multiple sclerosis (15, 16). EAE arises as a consequence of the breakdown of self-tolerance, induced by the immunization of susceptible animals with myelin-derived Ags emulsified in CFA or following adoptive transfer of T cell lines or clones specific for various myelin proteins (15, 17, 18). Our present data show that s.c. administration of LF 15-0195 is effective in inhibiting the induction of rat EAE and the progression of established disease. Analysis of cellular and humoral immune responses revealed that the beneficial effect of LF 15-0195 treatment is mediated by the induction of a long-lasting tolerance in MBP-specific autoaggressive CD4 T cells.
BENEFICIAL EFFECT OF LF 15-0195 ON RAT EAE

Materials and Methods

Rats

Eight- to 10-week-old male Lewis (LEW) rats were used in this study. These animals were obtained from the Center d’Elevage R. Janvier (Le Genest St. Isle, France) and maintained in our animal house facility under specific pathogen-free conditions. All procedures were in accordance with national regulations on animal experiments.

Induction, treatment, and clinical evaluation of EAE

To induce active EAE, LEW rats were injected in the hind footpads with 10 μg of myelin basic protein (MBP) from guinea pig emulsified in CFA containing 4 mg/ml heat-killed Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI). MBP was prepared in our laboratory as previously described (19). A total of 100 μl of MBP-CFA was divided equally between the rear footpads. To induce passive EAE, encephalitogenic T cells from MBP-immunized donors were adoptively transferred into naïve syngeneic recipients as previously described (20). Briefly, lymph node cells (popliteal and para-aortic) from MBP-immunized rats were collected 10–12 days after MBP sensitization and stimulated in vitro for 3 days with either MBP (2 μg/ml) or Con A (1 μg/ml). Viable T cells separated from dead cells by Ficoll-Hypaque were i.v. injected into syngeneic recipients. LF 15-0195 (Fournier Laboratories, Daix, France) was prepared in saline solution and adjusted at pH 7.2, and 300 μl was administered s.c. at the indicated regimen and concentration.

Control rats received either LF 15-0195 or saline without immunization with MBP. Animals were scored daily for clinical signs of disease on a severity scale ranging from 0 to 5: 0, normal; 1, limp tail; 2, hind limb weakness; 3, unilateral hind limb paralysis; 4, bilateral hind limb paralysis; and 5, bilateral hind limb paralysis and incontinence. Clinical signs determined to be between any of these stages were given an intermediate score. The results are presented as the mean cumulative score and/or the mean maximal score around day 15. The disease was severe and killed 100% of the animals, except those treated with a prolonged course of LF 15-0195 for 30 days, starting from the day of immunization, at two different doses (10 and 30 mg/kg for 15 days) (group 2 and 3) (21). The results obtained are summarized in Table I. All 16 LEW rats immunized with MBP and injected with saline developed typical clinical signs of acute EAE. The first clinical signs occurred 10–11 days after immunization, progressing to paraparesis, with maximum severity around day 15. The disease was severe and killed 50% of the animals. The surviving rats developed a self-limiting disease that lasted for ~1 wk (group 1). In contrast, LEW rats immunized with MBP and treated with a daily dose of 1 mg/kg LF 15-0195 for 7 days (group 2) or 15 days (group 3), starting from the day of MBP immunization, developed milder EAE compared with controls. The onset of disease was significantly delayed (p = 0.0006), and only 20% of the animals died from disease (groups 2 and 3). Since the animals from group 3 started to develop EAE 5 days after treatment withdrawal, we tested the effect of a prolonged treatment (30 days), starting from the day of immunization, at two different doses. A very significant reduction of incidence and severity of clinical EAE was obtained with a 30-day course of LF 15-0195 treatment at 1 mg/kg (group 5). Indeed, none of the animals died from disease, and only five animals among 19 developed delayed (19.3 ± 1.5 vs 11.5 ± 0.8; p = 0.0006) and mild (2.8 ± 1.5 vs 4.9 ± 0.3; p = 0.0001) clinical EAE compared with controls. The protected animals were still free of clinical signs 3 mo after treatment withdrawal. Treatment with 0.3 mg/kg for 30 days (group 4) was less efficient at preventing EAE than treatment with 1 mg/kg for the same period. Indeed, the severity and incidence of EAE were not significantly different from those of the
control animals; nevertheless, none of the six rats of this protocol died from disease. The control unimmunized LEW rats (group 6) injected with saline or LF 15-0195 (1 or 0.3 mg/kg) for 15 or 1 mo were free of disease and appeared healthy without any signs of side effects.

Animals with clinical EAE present inflammatory infiltrates in their CNS, but infiltration of leukocytes is not always associated with clinical signs of EAE (31, 32). Therefore, we analyzed whether the protection from EAE by LF 15-0195 is associated with inhibition of the development of inflammatory infiltrates in the CNS. Immunohistologic examination of the spinal cords of MBP-immunized LEW rats, treated or not with LF 15-0195 at 1 mg/kg for 15 days, shows a marked reduction in the magnitude of leukocyte infiltration in the protected animals. This reduction was observed for CD4 and CD8 T cells and macrophages, and there was a parallel reduction of MHC class II Ag expression on the microglia. Fig. 1A shows representative spinal cord sections in which leukocyte infiltration and MHC Ag class II expression are observed in diseased rats, but not in protected rats. These results were confirmed by the quantification of leukocyte infiltration in the spinal cord after purification using a Percoll gradient (Fig. 1B). The CNS of LF 15-0195-treated rats were still free of leukocyte infiltrates 20 days after treatment withdrawal (Fig. 1B), indicating that, unlike rats treated with other immunosuppressive drugs (33), LF 15-0195-protected rats did not develop relapsing disease after treatment withdrawal. In combination, our results show that LF 15-0195 treatment prevents active EAE, and that the protection afforded is associated with a markedly reduced encephalomyelitis, as evaluated histologically.

**LF 15-0195 treatment also reduces the severity of ongoing and passive EAE**

To study the effect of LF 15-0195 on the progression of ongoing EAE, daily treatment of MBP-immunized LEW rats with LF 15-0195 was initiated on day 7 (before appearance of EAE clinical signs) or day 11 (animals have at least a limp tail) after MBP immunization and continued for a period of 15 days. Table II shows that during this treatment, MBP-immunized LEW rats developed less severe clinical EAE (the maximal clinical score and mortality were significantly reduced) compared with control MBP-immunized LEW rats. Treatment with LF 15-0195 at 2 mg/kg proved more efficient than 1 mg/kg, and the effect of LF 15-0195 was more pronounced if started early, before the appearance of clinical EAE (day 7). These data suggest that LF 15-0195 may affect the pathogenicity of already differentiated effector lymphocytes. This is further supported by the effect of LF 15-0195 on adoptively transferred EAE. We determined that when \(25 \times 10^6\) MBP-stimulated immune lymph node cells were transferred, recipients developed classical EAE, with maximal clinical score of 3. Next, we tested the effect of LF 15-0195 treatment on passive EAE by injecting the recipients daily with 1 mg/kg of this drug for 10 days, starting the day before the transfer of encephalitogenic T cells. Fig. 2 shows that recipients exposed to LF 15-0195 for 10 days exhibited a statistically significant (\(p = 0.0032\)) decrease in EAE compared with control animals injected with the same effectors without LF 15-0195 treatment. Moreover, lymph node cells from MBP-immunized LEW rats when restimulated in vitro in the presence of LF 15-0195 had a reduced capacity to transfer EAE (data not shown). Altogether these data indicate that LF 15-0195 can block the pathogenic effect of differentiated autointerdigitating T cells and suggest that this drug may be used to inhibit the progression of established disease.

**Analysis of Ag-specific T cell responses in LF 15-0195-treated or untreated MBP-immunized LEW rats**

To investigate the mechanisms involved in prevention of EAE, we compared the proliferative capacity and the cytokine profiles of immune lymph node cells from MBP-immunized LEW rats, treated daily, or not, with LF 15-0195 for a total of 12 days from the day of MBP immunization. The proliferative response after MBP stimulation in vitro was similar in the two groups (Fig. 3A). The analysis of Ag-specific type 1 (IFN-γ and TNF-α) and type 2 (IL-4 and IL-10) cytokine production revealed that lymph node cells from LF 15-0195-treated animals produced significantly less IFN-γ (Fig. 3B) and IL-10 (Fig. 3C) and expressed low amounts of TNF-α mRNA (Fig. 3D) compared with lymph node cells from untreated MBP-immunized LEW rats. The expression of TGF-β (Fig. 3E) and IL-4 (Fig. 3F) mRNA by immune lymph node cells from protected and diseased rats is not significantly different. Taken together, these data demonstrate that although MBP-specific T cells proliferated equally well in vitro, the production of effector (IFN-γ and TNF-α) and regulatory (IL-10) cytokines was lower in LF 15-0195-treated compared with untreated rats. This difference was not observed when lymph node cells were stimulated with Con A (data not shown), suggesting that this treatment did not have a general immunosuppressive effect.

**Analysis of Ag-specific Ab responses in LF 15-0195-treated or untreated MBP-immunized LEW rats**

The sera obtained from MBP-immunized LEW rats, treated, or not, with LF 15-0195 for 30 days at 1 mg/kg, starting from the day of immunization, were analyzed for the presence of MBP-specific IgG. The anti-MBP IgG response was drastically reduced in the sera from LF 15-0195-treated animals (Fig. 4A), and this reduction

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**Table 1. LF 15-0195 treatment prevents actively induced EAE**

<table>
<thead>
<tr>
<th>Groups</th>
<th>MBP Immunization</th>
<th>Treatment Dosage and Duration</th>
<th>Incidence (%)</th>
<th>Day of Onset</th>
<th>Severity of Disease</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>Saline</td>
<td>16/16 (100)</td>
<td>11.5 ± 0.8*</td>
<td>4.9 ± 0.3*</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>LF 15-0195 (1 mg/kg, days 0–6)</td>
<td>5/5 (100)</td>
<td>14.2 ± 0.5</td>
<td>4.0 ± 1.2</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>LF 15-0195 (1 mg/kg, days 0–14)</td>
<td>5/5 (100)</td>
<td>20.0 ± 0.7</td>
<td>4.0 ± 1.2</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>LF 15-0195 (0.3 mg/kg, days 0–29)</td>
<td>6/6 (100)</td>
<td>11.0 ± 0.0</td>
<td>4.5 ± 0.8</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>LF 15-0195 (1 mg/kg, days 0–29)</td>
<td>5/9 (26)</td>
<td>19.3 ± 1.5</td>
<td>2.8 ± 1.5</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>Saline or LF 15-0195</td>
<td>0/11 (0)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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* LEW rats in groups 1–5 were immunized on day 0 with 10 μg of MBP emulsified in CFA. In group 6, the rats were not immunized.

* Delay between MBP immunization and onset of EAE.

* Severity of active EAE is represented by the mean of the maximal clinical score (±SD) of diseased rats.

* Statistical analyses: differences in day of onset and severity of active EAE between group 1 and groups 2, 3, and 5 are highly significant (\(p < 0.01\)). Group 1 vs group 4 is not significant.
FIGURE 1. LF 15-0195 treatment suppresses histological encephalomyelitis in MBP-immunized LEW rats. Spinal cords were removed from LEW rats that were immunized with MBP in CFA and treated, or not, with LF 15-0195, starting from the day of MBP immunization. A, The photographs show immunohistologic staining of spinal cord cryostat sections from a diseased rat (left panels) and a protected rat (right panels) after treatment with LF 15-0195 at 1 mg/kg for 15 days. The sections are stained for MHC class II Ag using OX6 mAb (upper panels) or for TCRαβ T cells using R73 mAb (lower panels). Magnification, ×400. B, The histograms show the absolute number of leukocytes that infiltrate the spinal cords of naïve (n = 3), protected (n = 3), and diseased (n = 9) rats on the indicated days after MBP immunization. Results are expressed as the mean value obtained from three to seven individual rats per group.

Concerns all the Ag-specific IgG subclasses tested (Fig. 4, B–D). The control nonimmunized LEW rats, treated, or not, with LF 15-0195, did not show any Ab response to MBP (data not shown).

Table II. Effect of LF 15-0195 treatment on the progression of actively induced EAE

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment Dosage and Duration</th>
<th>Incidence (%)</th>
<th>Day of Onsetb (mean ± SD)</th>
<th>Severity of Diseasec (mean ± SD)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>13/13 (100)</td>
<td>11.3 ± 0.8</td>
<td>5.0 ± 0.0f</td>
<td>33</td>
</tr>
<tr>
<td>2</td>
<td>LF 15-0195 (1 mg/kg, days 7–21)</td>
<td>5/5 (100)</td>
<td>11.4 ± 0.6</td>
<td>2.4 ± 1.5</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>LF 15-0195 (1 mg/kg, days 11–25)</td>
<td>5/5 (100)</td>
<td>12.2 ± 1.1</td>
<td>4.0 ± 0.7</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>LF 15-0195 (2 mg/kg, days 7–21)</td>
<td>1/4 (25)</td>
<td>11</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>LF 15-0195 (2 mg/kg, days 11–25)</td>
<td>4/4 (100)</td>
<td>11.0 ± 0.0</td>
<td>2.0 ± 2.0</td>
<td>75</td>
</tr>
</tbody>
</table>

* LEW rats in groups 1–5 were immunized on day 0 with 10 μg of MBP emulsified in CFA.
* Delay between MBP immunization and onset of EAE.
* Severity of active EAE is represented by the mean of the maximal clinical score (±SD) of diseased rats.
* Statistical analyses: differences in severity of EAE between group 1 and groups 2–5 are highly significant (p < 0.01).
equally well in vitro, the production of IL-10 and IFN-γ was lower in T cell lines obtained from protected rats compared with those obtained from diseased animals (data not shown). We also tested whether the in vitro stimulation of these lines in the presence of the Th1-promoting cytokine, IL-12, would increase their ability to transfer EAE. We observed that although IL-12 increases the ability of the T cell lines from protected rats to produce type 1 effector cytokines (data not shown), it does not unmask their reduced ability to transfer EAE (Fig. 6C). In contrast, this cytokine increases significantly the encephalogenicity of MBP-specific T cell lines obtained from diseased rats (Fig. 6C). Taken together, these data show that LF 15-0195 induces tolerance in MBP-specific CD4 T cells.

**FIGURE 4.** LF 15-0195 treatment reduces MBP-specific IgG responses. LEW rats were immunized with MBP in CFA and treated (■) with LF 15-0195 (1 mg/kg) for 30 days, starting from the day of immunization. IgG anti-MBP (A), IgG1 anti-MBP (B), IgG2a anti-MBP (C), and IgG2b anti-MBP (D) titers were measured by ELISA on days 5, 11, 17, 24, 31, and 39 after immunization. Results are expressed as absorbance at 450 nm of a pool of sera diluted 1/800 for total IgG and 1/1600 for IgG subclasses.

**FIGURE 5.** MBP-specific CD4 T cells from LF 15-0195-treated rats have a diminished capacity to transfer EAE. Lymph node cells from animals in which EAE was completely suppressed by LF 15-0195 treatment at 1 mg/kg, starting from the day of MBP challenge (■, n = 11 for A and B and n = 7 for C and D) or from control PBS-treated diseased rats (○, n = 4 for A and B and n = 4 for C and D) were adoptively transferred. Lymph nodes were removed on day 10 or 12 following MBP immunization, and cells were either cultured in vitro with MBP (200 μg/ml) or Con A (1 μg/ml) for 3 days. Viable MBP-stimulated leukocytes (10^6; A and B) or 10^9 viable Con A-stimulated lymphocytes (C and D) were injected i.v. into each naive syngeneic recipient. The results shown represent in A and C the mean clinical scores for paralysis of each experimental group and in B and D the mean total disease score of each experimental group. Results in A and B are from two independent experiments, and those in C and D are from one experiment. *, p < 0.05; **, p < 0.01 (by Mann-Whitney U test).
Results are derived from two independent experiments. C and in B specific T cell lines generated after day treatment is less effective than 0.3 mg/kg s.c. from the day of MBP immunization of LEW rats. This may explain their inability to induce CNS inflammation. In the present study we show that although immune lymph node cells from LF 15-0195-protected animals present Ag as efficiently as those from untreated animals to MBP-specific T cell lines (unpublished observations). These results suggest that the beneficial effect of LF 15-0195 on EAE may not be mediated by a defect in Ag presentation by professional APC or by induction of anergy in Ag-specific CD4 T cells.

In EAE the immunization with MBP elicits an autoimmune response toward myelin Ag, whereas autoreactive T lymphocytes and macrophages migrate into the CNS and produce inflammatory cytokines such as IFN-γ, TNF-α, and lymphotixin. These inflammatory cytokines have crucial roles in initiating and perpetuating CNS inflammation. In the present study we show that although immune lymph node cells from LF 15-0195-protected animals proliferate equally well in response to MBP in vitro, they produce significantly lower amounts of effector cytokines (IFN-γ and TNF-α) compared with those from untreated MBP-immunized LEW rats. This may explain their inability to induce CNS inflammation and to recruit other inflammatory leukocytes in the CNS. LF 15-0195 may therefore mediate some of its immunosuppressive properties by interfering with the ability of autoreactive T cells to differentiate into effector cytokine-producing cells. However, LF 15-0195 reduces the severity of both ongoing and passively induced EAE, suggesting that this compound also affects already differentiated pathogenic CD4 T cells. Whether this effect is direct or indirect remains to be elucidated.

Interestingly, our data show that LF 15-0195 treatment induces a stable and persistent tolerance in Ag-specific CD4 T cells. Indeed, the T cell lines obtained from LF 15-0195-treated animals, despite having been repeatedly restimulated in vitro in the absence of LF 15-0195, were unable to transfer severe EAE. Furthermore, the stimulation of these lines with IL-12, a key cytokine for the differentiation of T cells into Th1, did not render these lines pathogenic. Recently, it has been demonstrated in fully MHC-mismatched heart allograft transplantation that LF 15-0195 treatment induces graft-specific tolerance (38). This tolerance requires the presence of donor APCs during LF 15-0195 injections, indicating that Ag stimulation is required during LF 15-0195 treatment for promoting Ag-specific tolerance. Therefore, LF 15-0195 may suppress any B and T immune response in a specific manner provided the Ag is presented to the immune system during the treatment period. Our present results support and extend these conclusions to self-reactive lymphocytes. Indeed, the beneficial effect of LF 15-0195 in our study is not mediated by nonspecific immunosuppression, since the inhibition of effector cytokine production is observed only when immune lymph node CD4 T cells are stimulated with MBP, but not after stimulation with the T cell mitogen Con A. Similar results are obtained in another animal model of autoimmunity, experimental autoimmune myasthenia gravis (14). In the model of allo-transplantation, the tolerance induced by LF 15-0195 is mediated by Ag-specific regulatory T cells that express CD25, proliferated weakly in response to alloantigen and transfer...
tolerance to second syngeneic recipients (38). In contrast, there are several lines of evidence indicating that, at least during the first 2 wk of LF 15-0195 treatment, regulatory T cells are not involved in the protection from EAE: 1) CD4 T cells from protected and diseased rats express similar levels of CD25 and proliferate in a similar way; 2) in a cotransfer experiment, T cell lines from protected animals are unable to prevent the encephalitogenic anti-MBP T cells to transfer EAE (our unpublished observations); 3) the neutralization of TGF-β, a key regulatory cytokine, during LF 15-0195 treatment did not abrogate the protection (our unpublished observations); 4) MBP-specific CD4 T cells from LF 15-0195 treatment produced lower amounts of IL-10, a cytokine produced and involved in the suppressive effect mediated by regulatory T cells (39); and 5) LF 15-0195 reduces the severity of ongoing and passively induced EAE; this effect is observed after a short period of treatment not consistent with generation of regulatory T cells.

In conclusion, we demonstrated that the immunosuppressant LF 15-0195 can be used as a preventive and curative treatment in experimental autoimmune. The beneficial effect of this drug is accompanied by decreased Ag-specific T cell responses. Although the molecular mechanisms underlying the effect of LF 15-0195 have yet to be elucidated, our data suggest that LF 15-0195 is a promising therapeutic reagent for the treatment of human autoimmune diseases.

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References