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Intrathecal Fas Ligand Infusion Strengthens Immunoprivilege of Central Nervous System and Suppresses Experimental Autoimmune Encephalomyelitis[1]

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Fas ligand (FasL) is an essential molecule strongly expressed in some immunoprivileged sites, but is expressed at very low levels in normal CNS. In this study, acute experimental autoimmune encephalomyelitis (EAE) was induced in Lewis rats with guinea pig myelin basic protein. Intrathecal infusion of recombinant FasL before EAE onset dose dependently suppressed acute EAE and alleviated pathological inflammation in lumbosacral spinal cord. This treatment greatly increased apoptosis in CNS inflammatory cells, but did not inhibit systemic immune response to myelin basic protein. Systemic administration of a similar dose of rFasL was ineffective. In vitro, encephalitogenic T cells were highly sensitive to rFasL-induced cell death, and activated macrophages were also susceptible. In addition, in vitro rFasL treatment potentiated the immunosuppressive property of rat cerebrospinal fluid. We conclude that intrathecal infusion of rFasL eliminated the initial wave of infiltrating T cells and macrophages, and therefore blocked the later recruitment of inflammatory cells into CNS. Although Fas receptor expression was observed on spinal cord neurons, astrocytes, and oligodendrocytes, no damage to these cells or to the myelin structure was detected after rFasL infusion. ***The Journal of Immunology,*** **2002, 169: 1561–1569.**

Myelin basic protein (MBP)[3]-induced experimental autoimmune encephalomyelitis (EAE) in Lewis rats is a well-characterized model for acute CNS autoimmune inflammation (1). Between the onset and the peak of clinical EAE, numerous T cells and macrophages cross the blood-brain barrier and accumulate in the meningeal and perivascular areas. Many cells also infiltrate into the CNS parenchyma. CNS inflammation, which is prominent in lumbosacral spinal cord (LSSC), parallels the EAE symptoms in both time course and severity. The recovery from acute EAE is correlated with the apoptosis of inflammatory cells and the recession of inflammation in CNS (2).

In immunoprivileged sites, multiple local mechanisms suppress in situ the development of immunogenic inflammation (3). The CNS is a relatively immunoprivileged site. Although both the afferent and the efferent limbs of a CNS-targeting immune response are suppressed by local CNS mechanisms (4), grafts with significant histocompatibility differences are rejected from CNS (5), and inoculation of a high dose of bacillus Calmette-Guérin into the brain parenchyma also results in CNS inflammation (6). EAE is another example suggesting that strong immune responses to CNS Ags can overwhelm the protection from CNS immunoprivilege.

The initiation of autoimmune inflammation in CNS has been well described in adoptive transfer EAE. When activated encephalitogenic T cells are transferred into the host circulation, they first randomly distribute throughout the body. Some transferred cells can be detected in CNS within a few hours (7). However, the homing of hundreds to a thousand times more encephalitogenic T cells and the recruitment of numerous host T cells and macrophages into the CNS, which directly result in pathological CNS inflammation, start only hours before the EAE onset (8, 9). During the preclinical period, which lasts for 3–4 days, major events occur at the CNS perivascular space. Those initially infiltrating T cells make contact with perivascular macrophages (10) and small numbers of infiltrating macrophages (11), which may present CNS Ags released during normal turnover. This Ag-specific recognition not only retains the infiltrating T cells (7), but also results in the production of proinflammatory cytokines and chemokines, which are responsible for the later large-scale homing and recruitment of inflammatory cells into the CNS (12, 13). We hypothesized that elimination of initially infiltrating Ag-specific T cells and activated macrophages in CNS during this preclinical period could prevent the homing and recruitment of inflammatory cells, and therefore suppress acute EAE.

The local expression of Fas ligand (FasL) is essential for maintaining immunoprivilege in the eye (14), the testis (15), and the placenta (16) through the deletion of early infiltrating inflammatory cells. In relation to this, FasL is essential for activation-induced T cell death (17–19). In some experimental conditions, activated macrophages, B cells, and neutrophils are also vulnerable to FasL-induced apoptosis (20–22). In models of autoimmune arthritis (23) and thyroiditis (24), overexpression of FasL in tissues targeted by autoimmunity greatly reduced disease severity. However, transplantation of grafts that overexpress FasL sometimes resulted in neutrophil infiltration and accelerated rejection (25), suggesting a proinflammatory effect of FasL. An immunosuppressive microenvironment, as represented by the presence of TGF-β, may be necessary for FasL to fully express its anti-inflammatory functions (26).

In the recovery phase of EAE, infiltrating T cells, macrophages, and activated microglia up-regulate Fas and FasL expression, and

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[3] Abbreviations used in this paper: MBP, myelin basic protein; CSF, cerebrospinal fluid; dpi, days postimmunization; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; FasL, Fas ligand; GFAP, glial fibrillary acidic protein; LSSC, lumbosacral spinal cord; PI, propidium iodide.

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are highly prone to apoptosis (2, 27). This suggests that up-regulation of Fas/FasL system in CNS may be an endogenous mechanism to resolve CNS autoimmune inflammation. However, this up-regulation occurs when CNS inflammation is fully blown, and is not able to inhibit the initiation and progression of EAE. In contrast to other immunoprivileged sites, constitutive FasL expression is very low in CNS (28). Nevertheless, multiple immunosuppressive mechanisms of the normal CNS may provide an ideal environment for exogenously administered FasL to exert its anti-inflammatory function. We have studied whether intrathecal infusion of rFasL before EAE onset could potentiate CNS immunoprivilege and suppress acute EAE.

Materials and Methods

EAE induction and observation

Male Lewis rats with a body weight between 175 and 200 g were obtained from Charles River Canada (St. Constant, Quebec, Canada). The protocols for animal experiments were approved by the Animal Care Center, University of British Columbia. To actively induce EAE, each rat was immunized s.c. close to the inguinal lymph nodes with 100 μl MBP/CFA emulsion, which contained 50 μg guinea pig MBP (Sigma-Aldrich, St. Louis, MO) and 500 μg heat-inactivated mycobacteria tuberculosis (Difco, Detroit, MI). The rats were weighed and scored for EAE severity daily over 20 days postimmunization (dpi). The degrees of EAE severity were scored as follows: 0, no clinical symptoms; 0.5, incomplete tail paralysis; 1, complete tail paralysis; 2, unsteady gait or incomplete paraplegia; 3, complete paraplegia.

Intrathecal infusion in Lewis rats

Fourteen-day osmotic minipumps (ALZA, Mountain View, CA) were each filled with 200 μl artificial cerebrospinal fluid (CSF) (29) supplemented with 20 μg gentamicin. The pumps were connected to a 6.5-cm PE-10 tube (BD Biosciences, Sparks, MD) through an infusion switch that is included in the brain infusion kit (ALZA). To implant pumps, a small opening was made in the subarachnoid matter covering cisterna magna, and the PE-10 tube was inserted caudally into the subarachnoid space. The tip of PE-10 tube was close to T12 of rat spinal cord. The minipumps were then embedded s.c. between the scapulae. Most animals started to regain weight 3–4 days after surgery. On the sixth day after surgery, animals were immunized with MBP/CFA. At 7 dpi, the original 14-day minipumps were changed to 3-day minipumps to infuse either rFasL (Upstate Biotechnology, Lake Placid, NY) or control solution between 7 and 10 dpi. rFasL is a recombinant protein corresponding to the entire extracellular domain of human FasL (aa 103–281) plus a FLAG tail. For rFasL infusion, the 100 μl solution contained 125–700 ng rFasL, 0.14 trypsin inhibitory unit aprotinin (to inhibit rFasL degradation), 80 μg rat albumin (as a carrier protein), and 10 μg gentamicin in artificial CSF. For control infusion, the 100 μl solution was the same, only without rFasL.

Morphological techniques

Animals were sacrificed by i.p. Euthanyl injection, and transcardially perfused with 300 ml PBS. LSSC was dissected out and cut into nine segments roughly corresponding to the L1 to S3 segments of the spinal cord. These segments were immersed in TissueTek in a cryomold and were oriented with the rostral ends toward the bottom. They were fresh frozen in liquid nitrogen, and 10-μm frozen sections were prepared. Luxol fast blue staining, toluidine blue staining, and immunostaining were performed following the established protocols (30). ED1, OX-42, OX-19, and W3/13 Abs were obtained from Serotec (Oxford, U.K.). Rip Ab was from Developmental Studies Hybridoma Bank at University of Iowa (Iowa City, IA). SMI-32 Ab was from Sternberger Monoclonals (Lutherville, MD). Fas (A20) and glial fibrillary acidic protein (GFAP) (C-19) Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). TUNEL staining was performed according to the protocol supplied by Oncogene Research Products (San Diego, CA). To quantitate inflammation, the total numbers of inflammatory foci were counted in H&E-stained sections from all nine different levels of LSSC. An inflammatory focus was defined as the presence of a cluster of 20 or more aggregated mononuclear cells (31). The numbers of immunostaining and TUNEL staining positive cells were also quantitated in LSSC sections. Under $\times$400 magnification (the diameter of the view field was 0.375 mm), the view field was focused on the most medial ventral white matter area of the right side of spinal cord without including the meninges and the anterior medium fissure, and the total numbers of positively stained cells were counted from nine levels of LSSC. Spinal cord neurons were counted in the right side of the gray matter from nine levels of LSSC after SMI-32 immunostaining.

Cell culture

MBP-specific T cell lines were established according to the standard protocol (32). Briefly, monocytes from the draining lymph nodes of MBP-immunized Lewis rats were isolated on 9 dpi, and were stimulated in vitro with 20 μg/ml MBP for 72 h. The viable lymphoblasts were purified by Ficoll density centrifugation, and expanded with IL-2 (Sigma-Aldrich) for 5 days. Cells were maintained through additional cycles of MBP stimulation in the presence of gamma-irradiated syngenic thymocytes, followed by expansion with IL-2. After the second and third round of MBP stimulation, 1–2 $\times$ 10^6 T line cells were sufficient to transfer 3^0 EAE in naive animals. Inflammatory peritoneal macrophages were obtained according to the reported protocol (33). Briefly, 5% proteose peptone (Sigma-Aldrich) in saline (5 ml/100 g body weight) was injected i.p. into Lewis rats. The cells in peritoneal exudates were collected after 72 h. After 2-h incubation, nonadherent cells were removed by rinsing. Over 90% of adherent cells were identified as macrophages by Giemsa staining.

T cell proliferation assay and delayed-type hypersensitivity (DTH)

T cell proliferation assays were performed as described (32) in monocytes from draining lymph nodes, and [^{3}H]thymidine incorporation was measured 3 days after stimulation with 20 μg/ml MBP. DTH tests were performed on 12 dpi. A total of 50 μl MBP solution (0.75 μg/μl) was injected intradermally at the dorsal aspect of the right ear. The thickness of the right ear was measured five times both before injection and 24 h after injection. The averaged increase in ear thickness after injection was recorded as the DTH response to MBP. The injection of 50 μl saline solution as controls did not result in any thickness increase after 24 h.

Cell death assays

MTT assays were performed by adding 5 mg/ml MTT to cell cultures at 1/10 dilution. After incubation at 37°C for 3 h, the cells were collected. The purple crystals were dissolved in 200 μl isopropanol, and the cell debris was removed. MTT results represent the differences in absorption between 560 and 690 nm read on a microplate reader. Annexin V-FITC/propidium iodide (PI) staining was performed according to the BD PharMingen (San Diego, CA) protocol, and the samples were analyzed by flow cytometry within 1 h.

CSF collection from Lewis rats

Under anesthesia, the subarachnoid matter covering cisterna magna was exposed by dissection. The tip of a 30-gauge needle attached to a syringe was inserted into the subarachnoid space, and ~150–200 μl clear CSF was collected from each animal. The albumin levels in pooled CSF were lower than 1/1000 of rat serum albumin level.

Statistics

Data are presented in mean $\pm$ SEM. Two-sample t tests were used to compare the mean values between two groups. Values of $p < 0.05$ were regarded as statistically significant.

Results

Intrathecal infusion of rFasL suppresses clinical EAE and alleviates pathological inflammation in LSSC

The clinical manifestations of EAE with various treatment protocols are summarized in Table I. Starting from 10 or 11 dpi, nontreated and control-infused rats all developed severe EAE symptoms. There were no significant differences in EAE onset, peak EAE severity, or weight loss between the two groups. Therefore, the procedure of intrathecal infusion as well as the ingredients in control infusion did not interfere with EAE development. In contrast, intrathecal infusion of 175–700 ng rFasL between 7 and 10 dpi dose dependently reduced EAE incidence. In those rFasL-infused animals that developed EAE symptoms, the onset was significantly delayed, and most of them only had difficulty in tail movement for 1–3 days. The weight loss was also much milder. Interestingly, none of 10 Lewis rats infused with 700 ng rFasL

Table I. *Intrathecal infusion, but not systemic administration of rFasL, suppresses acute EAE in Lewis rats*

Groups[a]	No. of Animals	% EAE Incidence	EAE Onset (dpi)[b]	Peak EAE Score[b]	Weight Loss (g)[b]
Nontreated	10	100 (10/10)	10.5 ± 0.3	2.8 ± 0.2	42.1 ± 2.5
Control infusion i.t.	15	100 (15/15)	10.6 ± 0.2	2.9 ± 0.2	44.5 ± 2.1
rFasL 175 ng i.t.	8	63 (5/8)	12.2 ± 0.4[c]	1.3 ± 0.3[c]	28.0 ± 4.6[c]
rFasL 350 ng i.t.	15	20 (3/15)	12.3 ± 0.3[c]	0.8 ± 0.1[c]	22.3 ± 6.1[c]
rFasL 700 ng i.t.	10	0 (0/10)	N/A	N/A	N/A
rFasL 175 ng × 4 i.m.	9	100 (9/9)	10.1 ± 0.5	2.7 ± 0.2	40.4 ± 4.1
rFasL 175 ng × 4 i.v.	4	100 (4/4)	10.7 ± 0.5	2.8 ± 0.3	47.0 ± 7.1

[a] The rats in all groups were immunized with MBP/CFA, and observed over 20 dpi. Intrathecal infusion (i.t.) was given between 7 and 10 dpi. The i.m. and i.v. injections of rFasL (175 ng each time) were given twice daily on both 8 and 9 dpi.
[b] Data regarding EAE onset, EAE severity, and weight loss were obtained from animals that developed clinical EAE.
[c] $p < 0.001$ compared with the data from nontreated animals.

developed any EAE signs over 20 dpi. They moved actively, and their body weight increased similarly as in normal animals. Therefore, intrathecal infusion of rFasL before the expected EAE onset is able to suppress acute EAE in Lewis rats.

Pathological examinations were performed in animals that were nontreated, infused with control solution, and infused with 350 ng rFasL. H&E staining shows that control-infused and nontreated animals at the EAE peak (12 dpi) developed severe inflammation in LSSC (Fig. 1*A*). In contrast, minimal inflammation was observed in LSSC from rFasL-infused animals at 12 dpi (Fig. 1*B*). In animals that were infused with rFasL and developed mild EAE, the degree of inflammation in LSSC was also much milder, correlating well with the alleviated EAE symptoms. Because most inflammatory cells infiltrate into CNS between EAE onset and EAE peak, we quantitated the numbers of inflammatory foci in LSSC at both 10 and 12 dpi (Fig. 1*C*). Although no significant changes were observed at either time point after control infusion, no inflammatory focus could be observed at 10 dpi after rFasL infusion, and the numbers of inflammatory foci were reduced by an average of 90% at 12 dpi after rFasL infusion.

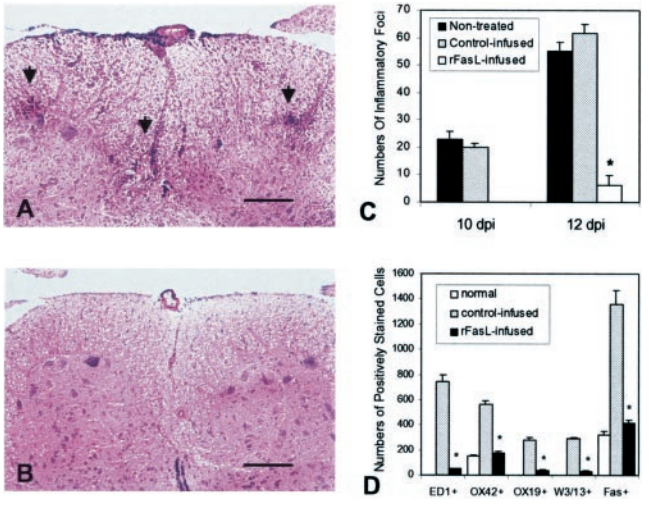

FIGURE 1. Intrathecal infusion of 350 ng rFasL between 7 and 10 dpi greatly alleviates pathological inflammation in LSSC at both 10 and 12 dpi. H&E staining in LSSC sections obtained at 12 dpi shows meningeal/parenchymal inflammation and many inflammatory foci (marked by arrows) in control-infused animals (*A*), but no obvious inflammation in rFasL-infused animals (*B*). Scale bar in *A* and *B* = 180 μm. Quantitation (*C*) shows that rFasL infusion, but not control solution, significantly reduced the numbers of inflammatory foci in LSSC at both 10 and 12 dpi. Quantitation of ED1, OX-42, OX-19, W3/13, and anti-Fas immunostaining (*D*) shows that rFasL infusion significantly reduced the infiltration of both T cells and macrophages/activated microglia, as well as Fas$^+$ cells, in LSSC obtained at 12 dpi. LSSC tissues from three to four animals were included in each group of *C* and *D* (*, $p < 0.001$).

T lymphocytes and macrophages/activated microglia represent most of the inflammatory cells in this EAE model (34). To examine whether rFasL infusion preferentially reduced one cell type, we performed immunostaining with multiple markers in LSSC sections from normal, control-infused, and rFasL-infused animals (Fig. 1*D*). ED1 Ab mainly stains for macrophages and activated microglia, but also weakly stains granulocytes. OX-42 Ab stains for microglia and some macrophages. OX-19 (anti-rat CD5) and W3/13 (anti-rat CD43) are markers mainly for T cells, but also stain some B cells and polymorphonuclear cells, respectively. Although no ED1, OX-19, or W3/13 positive cells were observed in normal LSSC sections, many OX-42$^+$ and Fas$^+$ cells were present. When the net increase in positive cell numbers from normal levels was compared between control-infused and rFasL-infused animals, we found that rFasL infusion reduced ED1$^+$, OX-42$^+$, OX-19$^+$, W3/13$^+$, and Fas$^+$ cells by 93, 95, 86, 90, and 93%, respectively. Because double immunostaining showed that both ED1$^+$ and OX-19$^+$ cells strongly express Fas receptors (data not shown), the reduction of Fas$^+$ cells is consistent with the reduction of infiltrating T cells and macrophages. Taken together, these data indicate that intrathecal infusion of rFasL greatly reduced the infiltration of inflammatory cells into the LSSC, and both T cells and macrophages were reduced to a similar level.

Intrathecal infusion of rFasL increases apoptosis in CNS inflammatory cells, but does not inhibit systemic immune response to MBP

To examine the mechanisms by which rFasL infusion suppresses clinical EAE and pathological inflammation in LSSC, we performed TUNEL staining to study whether rFasL infusion induced apoptosis in CNS-infiltrating inflammatory cells. Because there are great differences in the numbers of inflammatory cells in LSSC between control-infused and rFasL-infused animals, it is important to compare the percentages of apoptotic inflammatory cells between the two groups. In various EAE models, most apoptotic cells in the CNS are either T cells or macrophages/activated microglia (27, 35–37). In contrast, apoptosis of neurons, oligodendrocytes, or astrocytes in EAE is very rare. As detailed later, we could not detect any cytotoxicity to neurons, oligodendrocytes, or astrocytes in LSSC after rFasL infusion either morphologically or quantitatively. Therefore, most apoptotic cells should be T cells and macrophages/activated microglia in both control-infused and rFasL-infused animals. We counted the numbers of OX-19$^+$, ED1$^+$, and TUNEL$^+$ cells in serial LSSC sections, and calculated the percentages of apoptotic CNS inflammatory cells. As shown in Table

Table II. *rFasL infusion increased apoptosis in CNS inflammatory cells*

Groups[a]	OX-19^{+b}	ED1^{+b}	TUNEL^{+b}	TUNEL^{+} (%)[c]
Control infusion (10 dpi)	123.7 ± 7.8	171.7 ± 9.6	23.4 ± 3.5	8.0 ± 1.2
rFasL infusion (10 dpi)	9.7 ± 2.7	6.3 ± 2.4	8.0 ± 2.6	49.4 ± 2.7[d]
Control infusion (12 dpi)	280.0 ± 16.3	746.3 ± 54.2	166.0 ± 3.5	16.3 ± 0.7
rFasL infusion (12 dpi)	38.0 ± 4.9	51.3 ± 2.0	38.7 ± 3.2	43.5 ± 3.6[e]

[a] In each group, LSSC tissues were obtained from three animals at 10 or 12 dpi, as indicated. The dose of rFasL infusion was 350 ng.
[b] The numbers of positively stained cells were counted, as described in *Materials and Methods*, in serial sections.
[c] The percentage of TUNEL^{+} cell numbers among combined numbers of OX-19^{+} and ED1^{+} cells.
[d] $p < 0.001$.
[e] $p = 0.0017$.

II, although the absolute numbers of TUNEL^{+} cells were lower in rFasL-infused animals due to a great reduction in inflammation, rFasL infusion significantly increased the percentages of apoptotic inflammatory cells from 8.0 ± 1.2% to 49.4 ± 2.7% at 10 dpi, and from 16.3 ± 0.7% to 43.5 ± 3.6% at 12 dpi. These data indicate that rFasL infusion greatly induced apoptosis in infiltrating inflammatory cells in CNS. Although double labeling of TUNEL^{+} cells with various cell markers would be meaningful, we found that TUNEL staining, which requires the pretreatment with proteinase K for best results, markedly reduced positive cell numbers in the following immunostaining.

To determine whether intrathecally infused rFasL might drain out of the CNS and directly affect systemic immune function, we tested whether systemic administration of a similar dose of rFasL could suppress EAE (Table I). When a total of 700 ng rFasL was injected either i.m. or i.v. on 8 and 9 dpi, no significant changes in EAE manifestations were observed. This suggests that infused rFasL acted locally within the CNS to suppress EAE development. However, it is possible that a local effect of rFasL may induce systemic immune deviation (38), which suppresses EAE. We compared MBP-induced T cell proliferation and DTH responses in rFasL-infused and control-infused animals (Fig. 2). No significant differences were observed in T cell proliferation at either 10 or 12 dpi, or in DTH responses at 12 dpi. Therefore, it is unlikely that systemic immune deviation or other tolerance mechanisms were responsible for the suppression of acute EAE after rFasL infusion.

In vitro rFasL treatment induces death in encephalitogenic T cells and activated macrophages, and potentiates the immunosuppressive property of rat CSF

There are controversial results regarding whether activated T cells and macrophages are susceptible to FasL-induced apoptosis (39, 40) and whether soluble FasL is efficient in inducing apoptosis (41, 42). We studied in vitro whether encephalitogenic T cells and activated macrophages are susceptible to rFasL-induced cell death. We established MBP-specific T cell lines and found that encephalitogenic T cells obtained after MBP stimulation with gamma-irradiated thymocytes are highly susceptible to rFasL-induced cell death. After 16 h of treatment with rFasL ranging from 0 to 25, 50, 100, and 200 ng/ml, the percentages of T cells double negative for Annexin V-FITC and PI staining among T cells/thymocytes decreased monotonically from 36.9 to 13.0, 6.1, 3.3, and 1.8% (Fig. 3*A–H*). The blockade of rFasL-induced T cell death by Fas-Fc fusion protein suggests that the effect of rFasL is specific (Fig. 3*I*). In addition, rFasL-treated T cells showed the characteristic morphology of apoptosis (Fig. 3, *J* and *K*). Although 1–2 × 10^6 nontreated T line cells were sufficient to transfer 3^0 EAE in a naive rat, 200 ng/ml rFasL-treated T cells with even a doubled pretreatment cell number could not transfer EAE at all (data not shown).

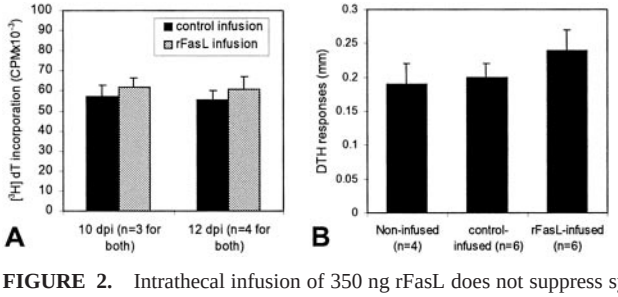

FIGURE 2. Intrathecal infusion of 350 ng rFasL does not suppress systemic immune response to MBP. No significant differences were found in comparing MBP-induced T cell proliferation between control-infused and rFasL-infused animals at either 10 or 12 dpi (*A*), or in comparing MBP-induced DTH responses among nontreated, control-infused, and rFasL-infused animals at 12 dpi ($p > 0.05$).

We next examined whether activated macrophages were susceptible to rFasL-induced cell death. Peritoneal inflammatory macrophages were first activated with 100 U/ml IFN-γ for 48 h, and then triggered with 200 ng/ml LPS. Because macrophages activated with this protocol are able to produce TNF-α, IL-12, and NO, and to exhibit tumoricidal activity in vitro (43, 44), they belong to the activated effector cells. rFasL, either alone or with the anti-FLAG Ab, was added at the same time as LPS. MTT assays show that rFasL treatment for 16 h dose dependently induced cell death in activated macrophages (Fig. 4). An average of 44% of macrophages was killed by 200 ng/ml rFasL treatment. When an anti-FLAG Ab (1.5 μg/ml; Upstate Biotechnology) was added together to cross-link rFasL via the FLAG tail, enhanced effects were observed, and ~72% of activated macrophages was eliminated with 200 ng/ml rFasL. These results suggest that while activated macrophages are not as sensitive as encephalitogenic T cells, a large proportion of activated macrophages is susceptible to rFasL-induced cell death.

In this study, we focus on testing our hypothesis that infusion of rFasL into the normal CSF before EAE onset could create a hostile environment for CNS Ag-directed T cells and macrophages to initiate CNS autoimmune inflammation. Because there was minimal inflammation in LSSC after intrathecal rFasL infusion, we think the CSF environment in which rFasL produced effect was close to the normal CSF. To mimic the in vivo situation, we examined how normal rat CSF, alone or in combination with rFasL, would affect MBP-induced proliferation of encephalitogenic T cells in vitro (Fig. 5). Different percentages (v/v) of CSF or HBSS (as controls) were included from the beginning of T cell proliferation experiments. Although 10–50% HBSS had no significant effect, the inclusion of 10–50% CSF dose dependently inhibited T cell proliferation. The inclusion of 50% CSF inhibited 76% of T cell proliferation. In addition, treatment with rFasL ranging from 25 to 100 ng/ml markedly enhanced the antiproliferative property of rat CSF. These data suggest

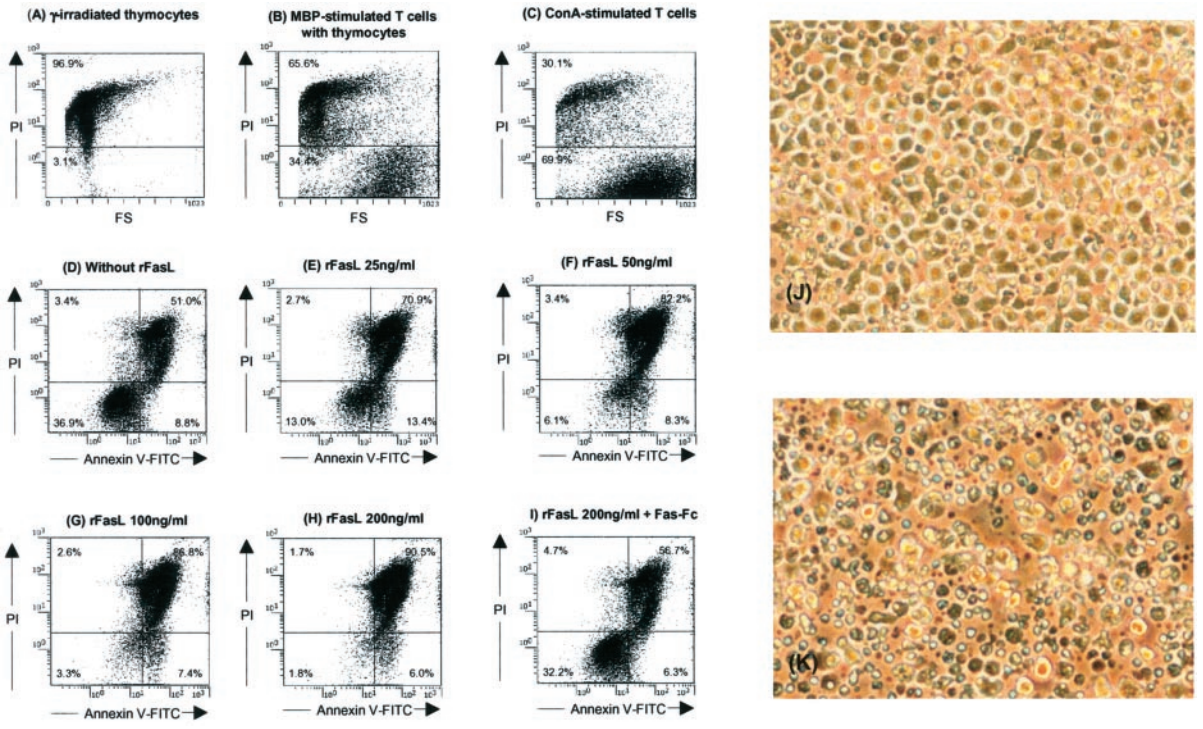

FIGURE 3. rFasL treatment in vitro induces apoptotic death in MBP-specific encephalitogenic T line cells. T line cells were obtained during the second or third round of MBP stimulation with gamma-irradiated thymocytes. rFasL treatment started after 24 h of MBP stimulation and lasted for 16 h. Annexin V-FITC/PI staining and flow cytometry show that rFasL dose dependently induced cell death in MBP-activated T blasts (*A–H*). Although T blasts in nontreated cultures could be distinguished from thymocytes in a forward scatter/side scatter plot, setting a gate specific for T cells was not feasible after rFasL treatment, as many dying or dead T cells moved out from the gated area and became mixed with thymocytes (data not shown). Because virtually all gamma-irradiated thymocytes were PI positive after 40 h in culture (*A*), most PI-negative cells in coculture were T blasts (*B*), which have similar cell size with T line cells collected after IL-2 expansion and then stimulated with 2 μg/ml Con A for 40 h (*C*). With the doses of rFasL ranging from 0 to 25, 50, 100, and 200 ng/ml, the percentages of MBP-stimulated T cells that were double negative for Annexin V-FITC and PI staining in T cell/thymocyte coculture decreased from 36.9 to 13.0, 6.1, 3.3, and 1.8% (*D–H*). When 5 μg/ml Fas-Fc fusion protein was added 0.5 h before 200 ng/ml rFasL treatment, rFasL-induced cell death was largely blocked. *I*, Under microscope, nontreated T blasts (*J*) were normal in morphology, but most cells treated with rFasL (*K*) were dead or dying. Many showed typical apoptotic morphology. These results were typical for three separate experiments.

that while CSF has a strong immunosuppressive function, exogenous FasL can greatly potentiate this suppression.

rFasL infusion does not damage neural cells or myelin in LSSC

Fas receptors have been detected on oligodendrocytes, astrocytes, and neurons in the normal CNS (36, 37). Applying double immunostaining of an anti-Fas Ab with SMI-32, Rip, and anti-GFAP Abs, we found that neurons, oligodendrocytes, and astrocytes in normal rat spinal cord constitutively express Fas receptors (Fig. 6,

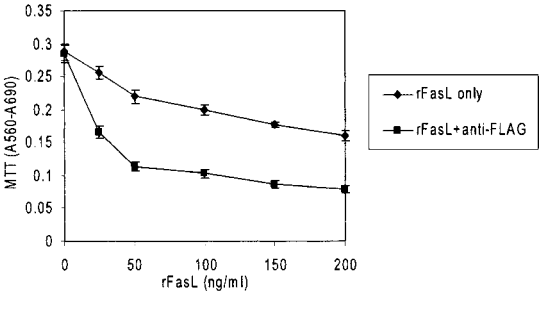

FIGURE 4. rFasL treatment dose dependently induces cell death in activated macrophages in vitro. Peritoneal inflammatory macrophages were primed with 100 U/ml IFN-γ for 48 h and then triggered with 200 ng/ml LPS. rFasL, either alone or in combination with anti-FLAG Ab (1.5 μg/ml), was added at the same time with LPS. MTT assay was performed 16 h later. rFasL alone dose dependently induced death in macrophages, and an enhanced effect was observed when rFasL was combined with anti-FLAG Ab. Anti-FLAG Ab alone did not show any cytotoxicity (data not shown). The results were typical for three separate experiments.

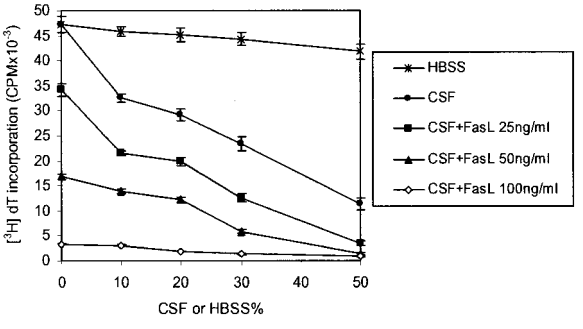

FIGURE 5. rFasL potentiates the immunosuppressive property of rat CSF. MBP-specific T line cells collected after IL-2 expansion were stimulated with 20 μg/ml MBP and gamma-irradiated thymocytes. A total of 0–50% (v/v) rat CSF or HBSS was included from the start of T cell proliferation experiment. rFasL was added 24 h later. [^{3}H]Thymidine was added after another 24 h, and cells were harvested 16 h later. Although 10–50% HBSS had no significant effect on T cell proliferation, the inclusion of 10–50% CSF dose dependently inhibited T cell proliferation. In addition, rFasL ranging from 25 to 100 ng/ml markedly enhanced the suppressive effect of rat CSF. The results were representative for three separate experiments.

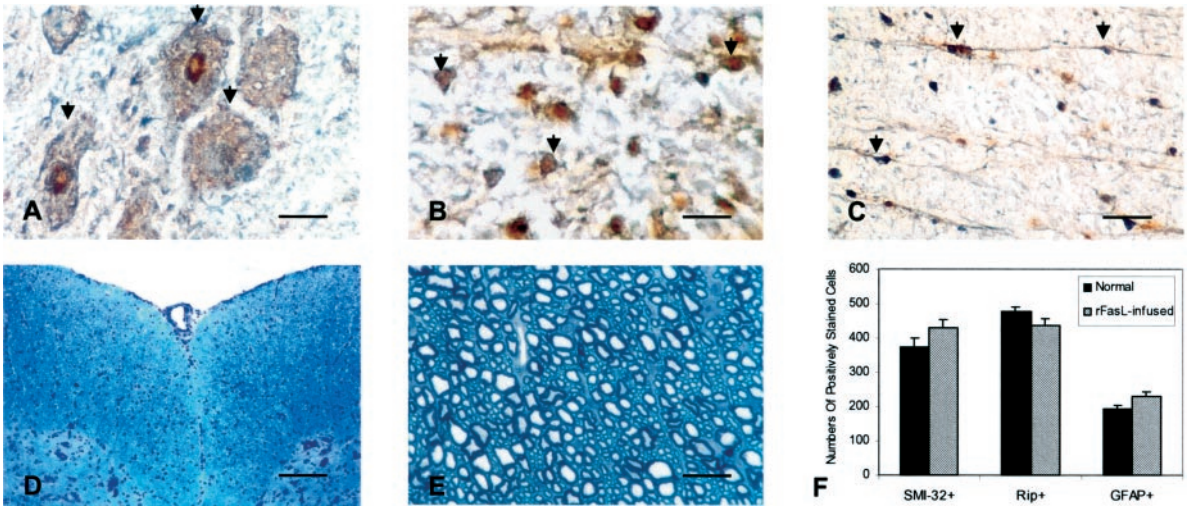

FIGURE 6. Intrathecal infusion of 700 ng rFasL does not damage myelin structure or neural cells in LSSC. Double immunostaining of an anti-Fas Ab with SMI-32 (*A*), Rip (*B*), and anti-GFAP (*C*) Abs shows that Fas receptors are constitutively expressed in neurons, oligodendrocytes, and astrocytes in normal spinal cord. Luxol fast blue staining in LSSC sections from rFasL-infused animals at 12 dpi (*D*) shows normal even myelin staining in white matter. Toluidine blue staining on semithin LSSC sections from rFasL-infused animals at 12 dpi (*E*) shows intact myelin structure and normal myelin thickness. Scale bars in *A–E* = 36, 18, 72, 72, and 18 μm, respectively. Quantitation of cells positive for Rip, SMI-32, and GFAP immunostaining in LSSC obtained at 12 dpi shows no significant changes in their numbers after rFasL infusion (*F*). There were three animals in each group ($p > 0.05$).

A–C). We therefore examined the potential cytotoxicity of infused rFasL in these neural cells, even though rFasL-infused rats appeared normal in behavior. In tissues obtained from 700 ng rFasL-infused animals at 12 dpi, Luxol fast blue staining shows normal even myelin staining in the white matter, and toluidine blue staining on semithin LSSC sections shows that myelin sheaths are intact and the myelin thickness is normal (Fig. 6, *D* and *E*). In addition, morphological examination (data not shown) and quantitation (Fig. 6*F*) of positively stained oligodendrocytes, neurons, astrocytes, and microglia in LSSC sections from rFasL-infused animals reveal no significant differences from normal animals (see Fig. 1*D* for microglia data). In conclusion, no toxic effect in spinal cord neural cells or the myelin structure was detected after the infusion of 700 ng rFasL.

Discussion

In this study, we found that MBP-specific encephalitogenic T line cells are highly sensitive to rFasL-induced cell death in vitro. Treatment with 200 ng/ml rFasL killed over 90% of these cells in 16 h. Soluble FasL includes the short form, which is shed from membrane-bound FasL by metalloprotease digestion, and the long form, which contains the entire extracellular domain of membrane-bound FasL (45). Although the short form soluble FasL retains little proapoptotic activity (41), the long form soluble FasL may be functional, especially when used at higher doses (42, 45). Our results show that rFasL, which belongs to the latter category, has a significant proapoptotic effect in encephalitogenic T cells. In addition, we found that the sensitivity of T line cells to rFasL depends on the MBP stimulation, because T line cells from the IL-2 expansion phase were resistant to rFasL (data not shown). This is consistent with reports showing that TCR occupancy is required for FasL-induced T cell death (46). It suggests that in vivo infiltrating encephalitogenic T cells may be deleted by rFasL when they are further activated by perivascular/infiltrating macrophages or by activated microglia in CNS.

Activated macrophages are the principal effector cells in EAE (47). In the preclinical period, macrophages are important APCs for T cells, and upon activation their production of proinflammatory cytokines and chemokines is important for the recruitment of inflammation cells into CNS. Our results show that a large portion of effector macrophages is susceptible to rFasL-induced cell death, although these cells are less sensitive than encephalitogenic T cells. We found that macrophages that were primed with IFN-γ only were resistant to rFasL (data not shown), suggesting that the susceptibility of macrophages to rFasL is also associated with their activation state and is correlated with their acquisition of effector functions. Because the interaction and mutual activation between infiltrating Ag-specific T cells and macrophages in CNS perivascular areas play a central role in the preclinical period, these data support our in vivo results showing intrathecal infusion of rFasL is able to stop the initiation of CNS autoimmune inflammation.

Accumulating evidence suggests that local suppression of the effector mechanisms of a CNS-directed immune response contributes to the CNS immunoprivilege. T cells infiltrating into CNS may lose the ability to produce IL-2 or to proliferate (48), and frequently undergo apoptosis (35, 49). Our results show that natural CSF from normal rats dose dependently suppressed MBP-induced T line cell proliferation in vitro. TGF-β and vasoactive intestinal peptide, which are present in normal CSF at effective concentrations (50), are able to suppress T cell proliferation (51, 52). Although TGF-β may be present in a latent form in CSF (50), a specific effect of TGF-β on Ag-presenting macrophages was demonstrated in the natural CSF (53). These macrophages may possibly generate a lower pH in the adjacent medium around them, and then activate the latent TGF-β in situ (54). A similar process may function to suppress T cell proliferation. There may be additional molecules in CSF, such as α-melanocyte-stimulating hormone (50), that also suppress T cell function. However, the suppression of T cell proliferation by normal CSF may be more restricted to Th1 cells. Natural CSF from normal humans and rabbits was found to enhance mycobacteria tuberculosis-induced proliferation of primed lymph node cells, although IFN-γ production in these cells was greatly reduced (50). It will be interesting to examine whether Th2 cells would respond to natural CSF by enhanced proliferation and cytokine secretion. Because CSF constantly communicates with perivascular space and meningeal areas, the strategic sites in which autoimmune T cells orchestrate the CNS autoimmune inflammation, and molecules smaller than 70

kDa may freely enter the extracellular fluid of the brain from the CSF (55), the immunosuppressive property of CSF may represent an important component of the CNS immunoprivilege. Our data further show that in vitro rFasL treatment greatly enhanced the immunosuppressive property of CSF, suggesting that infusion of rFasL into CSF may help to generate a more hostile environment for infiltrating T cells to initiate autoimmune inflammation in CNS.

Our data show that intrathecal infusion of rFasL before EAE onset dose dependently suppresses acute EAE and pathological inflammation in LSSC. This suppression was due to a local action of rFasL within the CNS, because rFasL infusion did not inhibit the systemic immune response to MBP, and systemic administration of a similar dose of rFasL was ineffective. When control-infused animals developed initial EAE symptoms at 10 dpi, many inflammatory foci and infiltrating T cells and macrophages could be observed in LSSC, but the percentage of apoptotic inflammatory cells was only ~8.0%. In contrast, in rFasL-infused animals, inflammatory cells and foci were rarely observed, but the percentage of apoptotic inflammatory cells was increased to 49.4%. When control-infused animals reached their EAE peak at 12 dpi, pathological inflammation in LSSC became much more severe, but the percentage of apoptotic inflammatory cells was still only 16.3%. At the same time point, the inflammation in LSSC of rFasL-infused animals was ~10 times lower, but the percentage of apoptotic inflammatory cells was 43.5%. These data suggest that rFasL infusion before EAE onset greatly increased apoptosis in initially infiltrating inflammatory cells, and therefore prevented the large-scale recruitment of inflammatory cells into CNS. Because intrathecal infusion of rFasL up-regulates the overall immunosuppressive properties of CNS and helps to maintain a largely inflammation-free CNS microenvironment, we conclude that this treatment is able to potentiate the immunoprivilege of the CNS.

Studies of EAE models in *lpr* and *gld* mice have shown that endogenous FasL is important in the recovery from EAE, but is not essential in EAE development or in demyelination (56–59). However, a pathogenic effect of endogenous FasL was observed in some strains of mice that are not highly susceptible to EAE induction (60–63). It has been proposed that FasL may be cytotoxic to oligodendrocytes in vivo, but direct evidence is still lacking (62). It is possible that in these EAE models endogenous FasL plays a more general proinflammatory role either systemically or within the CNS (64, 65). A recent study reported that intrathecal injection of anti-FasL Ab after EAE onset reduced EAE severity, but EAE relapse occurred earlier (66). These results support a potential dual role of endogenous FasL in EAE, although some concerns might exist as to whether intrathecally injected anti-FasL Ab may help to delete FasL-expressing T cells and activated macrophages/microglia within CNS through complement-mediated or cell-mediated cytotoxicity. In our current study, we did not observe any proinflammatory effect of intrathecal rFasL infusion. Several possibilities may explain the difference. It has been shown that soluble FasL has a much lower proinflammatory effect than membrane-bound FasL (42). Second, neutrophil infiltration in the Lewis rat EAE model is negligible, whereas it is significant in some mouse EAE models (67, 68). Endogenous FasL may have a chemotactic effect to recruit more neutrophils into the CNS (64). Third, CNS administration of rFasL before EAE onset allows rFasL to delete initially infiltrating inflammatory cells in a largely normal CNS microenvironment that is overall immunosuppressive. It will be important to determine how we can take advantage of the anti-inflammatory effect of FasL in preventing and treating CNS autoimmune diseases while blocking its potential proinflammatory effects. Although oligodendrocytes, astrocytes, and neurons in LSSC are positive in Fas immunostaining, we did not observe any cytotoxicity in neural cells after rFasL infusion. In EAE, FasL is highly expressed on CNS inflammatory cells, but apoptosis is only observed in some activated microglia among CNS neural cells (27, 36). This suggests that other CNS neural cells may resist the cytotoxicity of FasL even in an inflammatory milieu. However, more studies will be needed to define exactly whether FasL may be cytotoxic to CNS neural cells in certain conditions.

Further study is also required to determine whether rFasL infusion can induce brain-associated immune deviation (69) after acute EAE is suppressed, and secondly, whether rFasL infusion can suppress EAE when neutrophils are involved in CNS inflammation. Recently, there has been strong interest in developing gene therapy strategies to allow the long-term expression of cytokines in CNS through the CSF route (70, 71). Overexpression of FasL in allograft endothelium (72) and thyroid follicular cells (73) by genetic approaches has been reported to effectively prevent the immune attacks on these target tissues. It would be important to examine whether the long-term expression of rFasL in CSF might prevent or reduce the relapses of CNS autoimmune inflammation. Although we observed a substantial effect of intrathecal rFasL infusion in suppressing acute EAE, the time window of this treatment appears critical. Our preliminary data suggest that intrathecal infusion of rFasL (700 ng/animal) either during the incubation period (0–3 dpi) or right after EAE onset (10–13 dpi or 11–14 dpi) was largely ineffective (data not shown). This may be explained by the high sensitivity of encephalitogenic T cells to rFasL and the essential role of these T cells in initiating CNS autoimmune inflammation during the preclinical effector phase. However, experiments with higher rFasL doses, or the combination with potent protease inhibitors may reveal the role of rFasL infusion in treating ongoing CNS autoimmune inflammation.

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References

1. Wekerle, H., K. Kojima, J. Lannes-Vieira, H. Lassmann, and C. Linington. 1994. Animal models. *Ann. Neurol. 36:S47.*
2. McCombe, P. A., I. Nickson, Z. Tabi, and M. P. Pender. 1996. Apoptosis of Vβ8.2$^+$ T lymphocytes in the spinal cord during recovery from experimental autoimmune encephalomyelitis induced in Lewis rats by inoculation with myelin basic protein. *J. Neurol. Sci. 139:1.*
3. Streilein, J. W. 1993. Immune privilege as the result of local tissue barriers and immunosuppressive microenvironments. *Curr. Opin. Immunol. 5:428.*
4. Keane, R. W. 1997. Immunosuppression: CNS effects. In *Immunology of the Nervous System.* R. W. Keane and W. F. Hickey, eds. Oxford University Press, New York, p. 642.
5. Sloan, D. J., M. J. Wood, and H. M. Charlton. 1991. The immune response to intracerebral neural grafts. *Trends Neurosci. 14:341.*
6. Matyszak, M. K. 1998. Inflammation in the CNS: balance between immunological privilege and immune responses. *Prog. Neurobiol. 56:19.*
7. Hickey, W. F., B. L. Hsu, and H. Kimura. 1991. T-lymphocyte entry into the central nervous system. *J. Neurosci. Res. 28:254.*
8. Cross, A. H., B. Cannella, C. F. Brosnan, and C. S. Raine. 1990. Homing to central nervous system vasculature by antigen-specific lymphocytes. I. Localization of 14C-labeled cells during acute, chronic, and relapsing experimental allergic encephalomyelitis. *Lab. Invest. 63:162.*
9. Ludowyk, P. A., D. O. Willenborg, and C. R. Parish. 1992. Selective localization of neuro-specific T lymphocytes in the central nervous system. *J. Neuroimmunol. 37:237.*
10. Hickey, W. F., and H. Kimura. 1988. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. *Science 239:290.*
11. Subramanian, S., D. N. Bourdette, C. Corless, A. A. Vandenbark, H. Offner, and R. E. Jones. 2001. T lymphocytes promote the development of bone marrow-derived APC in the central nervous system. *J. Immunol. 166:370.*
12. Berman, J. W., M. P. Guida, J. Warren, J. Amat, and C. F. Brosnan. 1996. Localization of monocyte chemoattractant peptide-1 expression in the central nervous system in experimental autoimmune encephalomyelitis and trauma in the rat. *J. Immunol. 156:3017.*

13. Adamus, G., M. Machnicki, D. Amundson, K. Adlard, and H. Offner. 1997. Similar pattern of MCP-1 expression in spinal cords and eyes of Lewis rats with experimental autoimmune encephalomyelitis associated anterior uveitis. *J. Neurosci. Res. 50:531.*
14. Griffith, T. S., T. Brunner, S. M. Fletcher, D. R. Green, and T. A. Ferguson. 1995. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science 270: 1189.*
15. Bellgrau, D., D. Gold, H. Selawry, J. Moore, A. Franzusoff, and R. C. Duke. 1995. A role for CD95 ligand in preventing graft rejection. *Nature 377:630.*
16. Hunt, J. S., D. Vassmer, T. A. Ferguson, and L. Miller. 1997. Fas ligand is positioned in mouse uterus and placenta to prevent trafficking of activated leukocytes between the mother and the conceptus. *J. Immunol. 158:4122.*
17. Dhein, J., H. Walczak, C. Baumler, K. M. Debatin, and P. H. Krammer. 1995. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature 373:438.*
18. Brunner, T., R. J. Mogil, D. LaFace, N. J. Yoo, A. Mahboubi, F. Echeverri, S. J. Martin, W. R. Force, D. H. Lynch, C. F. Ware, et al. 1995. Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature 373:441.*
19. Ju, S. T., D. J. Panka, H. Cui, R. Ettinger, M. el-Khatib, D. H. Sherr, B. Z. Stanger, and A. Marshak-Rothstein. 1995. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. *Nature 373:444.*
20. Ashany, D., X. Song, E. Lacy, J. Nikolic-Zugic, S. M. Friedman, and K. B. Elkon. 1995. Th1 $CD4^+$ lymphocytes delete activated macrophages through the Fas/APO-1 antigen pathway. *Proc. Natl. Acad. Sci. USA 92:11225.*
21. Watanabe, D., T. Suda, and S. Nagata. 1995. Expression of Fas in B cells of the mouse germinal center and Fas-dependent killing of activated B cells. *Int. Immunol. 7:1949.*
22. Renshaw, S. A., S. J. Timmons, V. Eaton, L. R. Usher, M. Akil, C. D. Bingle, and M. K. Whyte. 2000. Inflammatory neutrophils retain susceptibility to apoptosis mediated via the Fas death receptor. *J. Leukocyte Biol. 67:662.*
23. Zhang, H., Y. Yang, J. L. Horton, E. B. Samoilova, T. A. Judge, L. A. Turka, J. M. Wilson, and Y. Chen. 1997. Amelioration of collagen-induced arthritis by CD95 (Apo-1/Fas)-ligand gene transfer. *J. Clin. Invest. 100:1951.*
24. Batteux, F., P. Lores, D. Bucchini, and G. Chiocchia. 2000. Transgenic expression of Fas ligand on thyroid follicular cells prevents autoimmune thyroiditis. *J. Immunol. 164:1681.*
25. Kang, S. M., D. B. Schneider, Z. Lin, D. Hanahan, D. A. Dichek, P. G. Stock, and S. Baekkeskov. 1997. Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction. *Nat. Med. 3:738.*
26. Chen, J. J., Y. Sun, and G. J. Nabel. 1998. Regulation of the proinflammatory effects of Fas ligand (CD95L). *Science 282:1714.*
27. Kohji, T., and Y. Matsumoto. 2000. Coexpression of Fas/FasL and Bax on brain and infiltrating T cells in the central nervous system is closely associated with apoptotic cell death during autoimmune encephalomyelitis. *J. Neuroimmunol. 106:165.*
28. French, L. E., M. Hahne, I. Viard, G. Radlgruber, R. Zanone, K. Becker, C. Muller, and J. Tschopp. 1996. Fas and Fas ligand in embryos and adult mice: ligand expression in several immune-privileged tissues and coexpression in adult tissues characterized by apoptotic cell turnover. *J. Cell Biol. 133:335.*
29. Kehne, J. H., D. I. Astrachan, E. Astrachan, J. F. Tallman, and M. Davis. 1986. The role of spinal cord cyclic AMP in the acoustic startle response in rats. *J. Neurosci. 6:3250.*
30. Zhu, B., G. R. Moore, T. J. Zwimpfer, L. F. Kastrukoff, J. K. Dyer, J. D. Steeves, D. W. Paty, and M. S. Cynader. 1999. Axonal cytoskeleton changes in experimental optic neuritis. *Brain Res. 824:204.*
31. Sobel, R. A., B. W. Blanchette, A. K. Bhan, and R. B. Colvin. 1984. The immunopathology of experimental allergic encephalomyelitis. I. Quantitative analysis of inflammatory cells in situ. *J. Immunol. 132:2393.*
32. Swanborg, R. H., and J. A. Stepaniak. 1996. Experimental autoimmune encephalomyelitis in the rat. In *Current Protocols in Immunology*, Vol. 3. J. E. Coligan, ed. John Wiley & Sons, New York, p. 15.2.1.
33. Naraba, H., M. Murakami, H. Matsumoto, S. Shimbara, A. Ueno, I. Kudo, and S. Oh-ishi. 1998. Segregated coupling of phospholipases A_2, cyclooxygenases, and terminal prostanoid synthases in different phases of prostanoid biosynthesis in rat peritoneal macrophages. *J. Immunol. 160:2974.*
34. Raine, C. S. 1984. Biology of disease: analysis of autoimmune demyelination: its impact upon multiple sclerosis. *Lab. Invest. 50:608.*
35. Schmied, M., H. Breitschopf, R. Gold, H. Zischler, G. Rothe, H. Wekerle, and H. Lassmann. 1993. Apoptosis of T lymphocytes in experimental autoimmune encephalomyelitis: evidence for programmed cell death as a mechanism to control inflammation in the brain. *Am. J. Pathol. 143:446.*
36. Bonetti, B., J. Pohl, Y. L. Gao, and C. S. Raine. 1997. Cell death during autoimmune demyelination: effector but not target cells are eliminated by apoptosis. *J. Immunol. 159:5733.*
37. Ouallet, J., N. Baumann, Y. Marie, and H. Villarroya. 1999. Fas system up-regulation in experimental autoimmune encephalomyelitis. *J. Neurol. Sci. 170:96.*
38. Streilein, J. W., and J. Y. Niederkorn. 1981. Induction of anterior chamber-associated immune deviation requires an intact, functional spleen. *J. Exp. Med. 153:1058.*
39. Inaba, M., K. Kurasawa, M. Mamura, K. Kumano, Y. Saito, and I. Iwamoto. 1999. Primed T cells are more resistant to Fas-mediated activation-induced cell death than naive T cells. *J. Immunol. 163:1315.*
40. Perlman, H., L. J. Pagliari, C. Georganas, T. Mano, K. Walsh, and R. M. Pope. 1999. FLICE-inhibitory protein expression during macrophage differentiation confers resistance to *fas*-mediated apoptosis. *J. Exp. Med. 190:1679.*
41. Tanaka, M., T. Itai, M. Adachi, and S. Nagata. 1998. Down-regulation of Fas ligand by shedding. *Nat. Med. 4:31.*
42. Hohlbaum, A. M., S. Moe, and A. Marshak-Rothstein. 2000. Opposing effects of transmembrane and soluble Fas ligand expression on inflammation and tumor cell survival. *J. Exp. Med. 191:1209.*
43. Hayes, M. P., J. Wang, and M. A. Norcross. 1995. Regulation of interleukin-12 expression in human monocytes: selective priming by interferon-γ of lipopolysaccharide-inducible p35 and p40 genes. *Blood 86:646.*
44. Lorsbach, R. B., W. J. Murphy, C. J. Lowenstein, S. H. Snyder, and S. W. Russell. 1993. Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing: molecular basis for the synergy between interferon-γ and lipopolysaccharide. *J. Biol. Chem. 268:1908.*
45. Suda, T., M. Tanaka, K. Miwa, and S. Nagata. 1996. Apoptosis of mouse naive T cells induced by recombinant soluble Fas ligand and activation-induced resistance to Fas ligand. *J. Immunol. 157:3918.*
46. Hornung, F., L. Zheng, and M. J. Lenardo. 1997. Maintenance of clonotype specificity in CD95/Apo-1/Fas-mediated apoptosis of mature T lymphocytes. *J. Immunol. 159:3816.*
47. Huitinga, I., N. van Rooijen, C. J. de Groot, B. M. Uitdehaag, and C. D. Dijkstra. 1990. Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. *J. Exp. Med. 172:1025.*
48. Irani, D. N., K. I. Lin, and D. E. Griffin. 1997. Regulation of brain-derived T cells during acute central nervous system inflammation. *J. Immunol. 158:2318.*
49. Pender, M. P., P. A. McCombe, G. Yoong, and K. B. Nguyen. 1992. Apoptosis of $\alpha\beta$ T lymphocytes in the nervous system in experimental autoimmune encephalomyelitis: its possible implications for recovery and acquired tolerance. *J. Autoimmun. 5:401.*
50. Taylor, A. W., and J. W. Streilein. 1996. Inhibition of antigen-stimulated effector T cells by human cerebrospinal fluid. *Neuroimmunomodulation 3:112.*
51. Cousins, S. W., M. M. McCabe, D. Danielpour, and J. W. Streilein. 1991. Identification of transforming growth factor-β as an immunosuppressive factor in aqueous humor. *Invest. Ophthalmol. Visual Sci. 32:2201.*
52. Taylor, A. W., J. W. Streilein, and S. W. Cousins. 1994. Immunoreactive vasoactive intestinal peptide contributes to the immunosuppressive activity of normal aqueous humor. *J. Immunol. 153:1080.*
53. Wilbanks, G. A., and J. W. Streilein. 1992. Fluids from immune privileged sites endow macrophages with the capacity to induce antigen-specific immune deviation via a mechanism involving transforming growth factor-β. *Eur. J. Immunol. 22:1031.*
54. Streilein, J. W., B. R. Ksander, and A. W. Taylor. 1997. Immune deviation in relation to ocular immune privilege. *J. Immunol. 158:3557.*
55. Nicholson, C., and E. Sykova. 1998. Extracellular space structure revealed by diffusion analysis. *Trends Neurosci. 21:207.*
56. Dittel, B. N., R. M. Merchant, and C. A. Janeway, Jr. 1999. Evidence for Fas-dependent and Fas-independent mechanisms in the pathogenesis of experimental autoimmune encephalomyelitis. *J. Immunol. 162:6392.*
57. Sabelko-Downes, K. A., A. H. Cross, and J. H. Russell. 1999. Dual role for Fas ligand in the initiation of and recovery from experimental allergic encephalomyelitis. *J. Exp. Med. 189:1195.*
58. Suvannavejh, G. C., M. C. Dal Canto, L. A. Matis, and S. D. Miller. 2000. Fas-mediated apoptosis in clinical remissions of relapsing experimental autoimmune encephalomyelitis. *J. Clin. Invest. 105:223.*
59. Liu, T. S., B. Hilliard, E. B. Samoilova, and Y. Chen. 2000. Differential roles of Fas ligand in spontaneous and actively induced autoimmune encephalomyelitis. *Clin. Immunol. 95:203.*
60. Sabelko, K. A., K. A. Kelly, M. H. Nahm, A. H. Cross, and J. H. Russell. 1997. Fas and Fas ligand enhance the pathogenesis of experimental allergic encephalomyelitis, but are not essential for immune privilege in the central nervous system. *J. Immunol. 159:3096.*
61. Waldner, H., R. A. Sobel, E. Howard, and V. K. Kuchroo. 1997. Fas- and FasL-deficient mice are resistant to induction of autoimmune encephalomyelitis. *J. Immunol. 159:3100.*
62. Malipiero, U., K. Frei, K. S. Spanaus, C. Agresti, H. Lassmann, M. Hahne, J. Tschopp, H. P. Eugster, and A. Fontana. 1997. Myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis is chronic/relapsing in perforin knockout mice, but monophasic in Fas- and Fas ligand-deficient *lpr* and *gld* mice. *Eur. J. Immunol. 27:3151.*
63. Okuda, Y., C. C. Bernard, H. Fujimura, T. Yanagihara, and S. Sakoda. 1998. Fas has a crucial role in the progression of experimental autoimmune encephalomyelitis. *Mol. Immunol. 35:317.*
64. Ottonello, L., G. Tortolina, M. Amelotti, and F. Dallegri. 1999. Soluble Fas ligand is chemotactic for human neutrophilic polymorphonuclear leukocytes. *J. Immunol. 162:3601.*
65. Rescigno, M., V. Piguet, B. Valzasina, S. Lens, R. Zubler, L. French, V. Kindler,

J. Tschopp, and P. Ricciardi-Castagnoli. 2000. Fas engagement induces the maturation of dendritic cells (DCs), the release of interleukin (IL)-1β, and the production of interferon γ in the absence of IL-12 during DC-T cell cognate interaction: a new role for Fas ligand in inflammatory responses. *J. Exp. Med. 192:1661*.

66. Okuda, Y., S. Sakoda, H. Fujimura, S. Nagata, T. Yanagihara, and C. C. Bernard. 2000. Intrathecal administration of neutralizing antibody against Fas ligand suppresses the progression of experimental autoimmune encephalomyelitis. *Biochem. Biophys. Res. Commun.* 275:164.
67. Moore, G. R., R. M. McCarron, D. E. McFarlin, and C. S. Raine. 1987. Chronic relapsing necrotizing encephalomyelitis produced by myelin basic protein in mice. *Lab. Invest. 57:157.*
68. McColl, S. R., M. A. Staykova, A. Wozniak, S. Fordham, J. Bruce, and D. O. Willenborg. 1998. Treatment with anti-granulocyte antibodies inhibits the effector phase of experimental autoimmune encephalomyelitis. *J. Immunol. 161:6421.*
69. Wenkel, H., J. W. Streilein, and M. J. Young. 2000. Systemic immune deviation in the brain that does not depend on the integrity of the blood-brain barrier. *J. Immunol. 164:5125.*
70. Martino, G., R. Furlan, G. Comi, and L. Adorini. 2001. The ependymal route to the CNS: an emerging gene-therapy approach for MS. *Trends Immunol. 22:483.*
71. Furlan, R., E. Brambilla, F. Ruffini, P. L. Poliani, A. Bergami, P. C. Marconi, D. M. Franciotta, G. Penna, G. Comi, L. Adorini, and G. Martino. 2001. Intrathecal delivery of IFN-γ protects C57BL/6 mice from chronic-progressive experimental autoimmune encephalomyelitis by increasing apoptosis of central nervous system-infiltrating lymphocytes. *J. Immunol. 167:1821.*
72. Sata, M., Z. Luo, and K. Walsh. 2001. Fas ligand overexpression on allograft endothelium inhibits inflammatory cell infiltration and transplant-associated intimal hyperplasia. *J. Immunol. 166:6964.*
73. Tourneur, L., B. Malassagne, F. Batteux, M. Fabre, S. Mistou, E. Lallemand, P. Lores, and G. Chiocchia. 2001. Transgenic expression of CD95 ligand on thyroid follicular cells confers immune privilege upon thyroid allografts. *J. Immunol. 167:1338.*