Dual Role for Fas Ligand in the Initiation of and Recovery from Experimental Allergic Encephalomyelitis

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Summary

We have previously demonstrated a role for Fas and Fas ligand (FasL) in the pathogenesis of experimental allergic encephalomyelitis (EAE). However, using an active induction paradigm we could not distinguish between FasL expressed on activated $CD4^+$ T cells from that expressed on other inflammatory or resident central nervous system (CNS) cells. To address this issue, we have conducted reciprocal adoptive transfer experiments of nontransgenic or myelin basic protein–specific T cell receptor transgenic wild-type, *lpr*, or *gld* lymphocytes into congenic wild-type, *lpr*, and *gld* hosts. We found that FasL expressed on donor cells is important for the development of EAE, as FasL-deficient lymphocytes transfer attenuated disease. Furthermore, Fas expressed in the recipient animals is important for the progression of EAE, as clinical signs of disease in *lpr* recipients were dramatically attenuated after transfer of either wild-type or *lpr* T cells. Surprisingly, these experiments also identified CNS cells as a source of functional FasL. Host-derived FasL appears to be especially important in the recovery from EAE, as many *gld* recipients of wild-type lymphocytes develop prolonged clinical signs of disease. Thus it appears that FasL plays distinct roles in EAE during the initiation of and recovery from disease.

Key words: autoimmunity • inflammation • T cell regulation • demyelinating disease • apoptosis

Experimental allergic encephalomyelitis (EAE)[1] is an animal model of the human demyelinating disease multiple sclerosis (MS). EAE can be induced in several rodent species by either active immunization with myelin components or passive transfer of activated, myelin-specific $CD4^+$ T cells (1, 2) of the Th1 (3, 4) but not Th2 subset into naive mice. The histological hallmark of EAE is a perivascular infiltrate comprised primarily of macrophages and T lymphocytes, which manifests itself clinically as a predictable course of ascending paralysis. Although it is thought that MS and EAE are autoimmune in nature, the precise target(s) and mechanism(s) of myelin disruption remain unknown.

Fas (CD95/APO-1) and Fas ligand (FasL; CD95L) are type I and type II transmembrane proteins and members of the TNF/nerve growth factor receptor and TNF families of proteins, respectively (5). Several recent studies have shown that expression of Fas and its ligand are elevated in MS lesions, implicating these molecules as potential effectors of this disease (6–8). Functional Fas is also expressed on myelin basic protein (MBP)-specific T lymphocytes isolated from MS patients (9, 10), suggesting that Fas-mediated death could contribute to the regulation of auto (central nervous system [CNS])-reactive T cell expansion in this disease as well.

We (11) and others (12, 13) have demonstrated that defective expression of Fas (*lpr*) or FasL (*gld*) dramatically ameliorates the clinical signs of MBP- or myelin oligodendrocyte glycoprotein–induced EAE in B10.PL or C57BL/6 mice, respectively. The mitigation of disease by the *lpr* and *gld* mutations does not result from abnormalities in the production of MBP-specific Th1 T cells, the development of a Th1-mediated immune response in vivo, or the infiltration of inflammatory cells into the CNS (11). These data implicated a role for Fas in the pathogenesis of EAE. However, using an active induction paradigm we could not distinguish between FasL expressed on activated $CD4^+$ T cells from that expressed on other inflammatory or resident CNS cells. In addition, any Fas-dependent regulatory function involved in the remission of actively induced EAE would have been masked by the dominant inhibitory effect of the *lpr* and *gld* mutations during the initiation and early progression of disease.

In this paper we have used an adoptive transfer model of EAE to address these issues in animals deficient in Fas or

[1]*Abbreviations used in this paper:* B6, C57BL/6; CNS, central nervous system; EAE, experimental allergic encephalomyelitis; FasL, Fas ligand; GVHD, graft-versus-host disease; MBP, myelin basic protein; MS, multiple sclerosis; TUNEL, TdT-mediated dUTP nick-end labeling.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/99/04/1195/11 $2.00
Volume 189, Number 8, April 19, 1999 1195–1205
http://www.jem.org

FasL. The data indicate that Fas expression in the host contributes to the pathogenesis of EAE. Furthermore, these data indicate that FasL is playing a dual role as an effector and as a regulator of disease via its interactions with Fas expressed on CNS elements and on infiltrating T lymphocytes, respectively.

Materials and Methods

Antigens and Animals. MBP was prepared from guinea pig spinal cords (Keystone Biologicals) as previously described (14). C57BL/6 (B6, H-2^b), B6Smn.MRL-*Fas^{lpr}* (B6.*lpr*, H-2^b), B6Smn.C3H-*Fasl^{gld}* (B6.*gld*, H-2^b), and B10.PL (H-2^u) mice were obtained from The Jackson Laboratory or produced in our own breeding colony from breeding stock obtained from The Jackson Laboratory. The congenic B10.PL.*Fas^{lpr}* and B10.PL.*Fasl^{gld}* mice were produced in our facility by backcrossing the mutations from the C57BL/6 background (B6.*lpr* or B6.*gld*) onto B10.PL for six generations. Because C57BL/10 and C57BL/6 are closely related substrains (15, 16) and neither mutation is linked to the MHC locus (MHC = chromosome 17, *lpr* = chromosome 19, *gld* = chromosome 1), transfer of the mutations from congenic C57BL/6 animals to C57BL/10.PL produces essentially congenic mutations on the B10.PL background. Mutant animals backcrossed one to six generations have behaved similarly in our active induction experiments (i.e., developed ameliorated clinical signs of EAE compared with B10.PL mice), indicating that the B10.PL.*Fas^{lpr}* and B10.PL.*Fasl^{gld}* mice are essentially congenic with B10.PL animals. MBP-specific TCR transgenic mice on a B10.PL background (H-2^u; MBP-1; provided by Hugh McDevitt, Stanford University, CA) (17) were crossed with B10.PL.*Fas^{lpr}* and B10.PL.*Fasl^{gld}* animals to produce MBP-specific TCR transgenic mice homozygous for the *lpr* or *gld* allele. Animals were screened for the MBP-specific TCR transgene by dual labeling PBLs with 10 μg/ml FITC-conjugated anti-CD4 (H129.19; PharMingen) and biotinylated anti-Vβ8.1,8.2 (MR5-2; PharMingen). PE-conjugated streptavidin (Southern Biotechnology Associates) was used as secondary reagent. Unless indicated otherwise, all experiments were begun with animals that were 6–8 wk of age and, in most instances, were completed before they had reached 12 wk of age. Animals were housed under specific pathogen–free conditions at the Washington University School of Medicine facility according to Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

Adoptive Transfer of EAE. Draining lymph nodes and spleens were removed from donor mice 10–12 d after subcutaneous immunization with 400 μg gpMBP and 60 μg *Mycobacterium tuberculosis* (H37RA; Difco Labs.) in IFA (Calbiochem). Single cell suspensions were treated with red blood cell lysis buffer (Sigma Chemical Co.) and washed, and 4–5 × 10^6 cells/ml were cultured with 20–25 μg/ml gpMBP in 6-well plates for 4 d. On day 4, cells were harvested and resuspended in HBSS. 5–8 × 10^7 viable cells were injected intravenously into 8-wk-old recipients (H-2^u) that had been sublethally irradiated (350 rads) 3–6 h previously. Recipient mice were also administered 200 ng pertussis toxin (List Biologicals) intravenously on days 0, 3, and 7. Cells isolated from donor mice immunized with CFA alone were not viable after 4 d in culture with gpMBP and therefore were not transferred. Instead, control mice were sublethally irradiated and administered all three doses of pertussis toxin. For the transfer of MBP-specific TCR transgenic cells, lymph nodes and spleens were removed from transgenic mice and single cell suspensions were depleted of red blood cells and cultured for 2 d in 50 ml of medium containing 10 μg/ml gpMBP in a T75 flask. On the second day, one-half of the medium was removed, replaced with fresh medium plus antigen, and incubated for an additional 2 d. Cells were harvested and resuspended in HBSS. 10^7 viable cells were injected intravenously into 6-8 wk old recipients (H-2^u) that had been sublethally irradiated (450R) 3-6 h previously. Pertussis toxin was not administered. Clinical signs of disease were monitored daily and were graded as follows: 0, normal; 0.5, partial loss of tail tonicity, assessed by inability to curl distal end of tail; 1, complete loss of tail tonicity; 2, mild to moderate hindlimb weakness or ataxia; 3, partial hindlimb paralysis; 4, complete hindlimb paralysis; 5, moribund. Moribund animals were killed. Aliquots of the cells transferred were stained with 10 μg/ml FITC-conjugated anti-CD4, anti-Lyt-2 (Becton Dickinson) or anti-CD19 (1D3; PharMingen), purified anti-CD11b (M1/70; PharMingen), and/or biotinylated anti-Vβ8.1, 8.2. FITC-conjugated goat anti–rat (1:40), and PE-conjugated streptavidin (1:300) were used as secondary reagents (Southern Biotechnology Associates).

Histological Analysis. CNS tissues removed from mice perfused with buffered 2.5% glutaraldehyde were postfixed in 1% osmium tetroxide (Electron Microscopy Sciences), dehydrated through graded alcohols and embedded in EMBED 812 (EMS) as previously described (18). 1 μm sections taken from each level of the CNS (optic nerves, cerebrum, cerebellum, brainstem, and cervical, thoracic, lumbar, and sacral spinal cord regions) were placed on glass slides, stained with toluidine blue, and assessed blindly using a published scoring system from 0 to 5 for inflammation, demyelination, and axonal necrosis (19).

Adoptive Transfer of B6 anti-B10.PL Cells. Splenocytes from B6 mice were cultured for 5 d with an equal number of irradiated B10.PL splenocytes in 50 ml of medium in a T75 flask (~3–6 × 10^8 B6 splenocytes/flask). On day 5, viable cells were recovered by Ficoll-Histopaque-1077 (Sigma Chemical Co.) density centrifugation, washed twice, and resuspended in HBSS. 5 × 10^7 viable cells were injected intravenously into 8-wk-old B10.PL or B10.PL.*Fas^{lpr}* recipients that had been sublethally irradiated (350 rads) 3–6 h previously. Control mice were sublethally irradiated, but were not given B6 anti-B10.PL cells. On days 1, 3, and 5 after transfer, control and injected animals were killed for analysis. Tissue samples from spleen, liver, lung, and small intestine were removed from mice and embedded in OCT (Miles, Inc.) for immunohistochemical analysis.

Immunohistochemistry. 9-μM frozen sections were placed on Superfrost Plus slides (Fisher Scientific Co.) and fixed in acetone for 10 min. Sections were stained with the appropriate Vectastain ABC-Elite kit (Vector Labs, Inc.) according to the manufacturer's instructions. In brief, sections were blocked with normal serum for 30 min and then incubated with primary antibody at 4°C overnight. Sections were washed twice and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min. Slides were then incubated with diluted ABC reagent for 30 min. Peroxidase reaction was developed using the VIP Vectastain substrate kit. Antibodies and dilutions used were biotinylated anti-K^b (1:50; PharMingen) and biotinylated anti-L^d (30-5-7) (1:100; provided by Dr. Ted Hansen, Washington University, St. Louis, MO).

TUNEL Assay and Fluorescent Immunolabeling. Mice were anesthetized with methoxyfluorane and perfused with saline. CNS tissues were removed and embedded in OCT. 9-μM frozen sections were placed on Superfrost Plus slides, fixed in 1% paraformaldehyde for 5 min, and permeabilized in 100% ethanol for 5 min. TUNEL (TdT-mediated dUTP nick-end labeling) staining was conducted using a modification of a previously published

protocol (20). In brief, slides were incubated with 0.3 U/μl terminal transferase (Boehringer Mannheim) and Cy3-labeled dUTP (Amersham Pharmacia Biotech) in TdT buffer (30 mM Tris HCl, pH 7.3, 140 mM sodium cacodylate, and 1 mM cobalt chloride) at 37°C for 1 h. The reaction was terminated in buffer containing 300 mM sodium chloride and 30 mM sodium citrate. After washing in PBS, sections were blocked with normal rabbit serum for 30 min and then incubated with either anti-CD4 (GK1.5; 1:1,000; Becton Dickinson) or anti-CD11b (M1/70; 1:10,000; PharMingen) at 4°C overnight. Slides were washed twice and incubated with biotinylated rabbit anti–rat secondary antibody (1:200; Vector Labs) for 30 min. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol for 30 min. Slides were then processed using the tyramide signal amplification (TSA)-direct kit (NEN Life Sciences) according to the manufacturer's instructions, counterstained with Hoechst 33258 (Polysciences, Inc.) and mounted in Fluoromount-G (Southern Biotechnology Associates). $TUNEL^+$ cells, $CD4^+$ or $CD11b^+$ cells, and double-labeled cells were counted in two to four inflammatory lesions per mouse using two coded slides per lesion. For some experiments, "nests" of >10 immediately adjacent $CD4^+$ cells were counted in four to five representative lower spinal cord cross-sections per mouse.

Results

We (11) and others (12, 13) have previously shown that both the *lpr* and *gld* mutations ameliorate the clinical signs of EAE actively induced in H-2^u or H-2^b mice by immunization with MBP or myelin oligodendrocyte glycoprotein, respectively. We have also demonstrated that these mutations do not affect the production of MBP-specific Th1 T cells, the development of a Th1-mediated immune response in vivo, or the infiltration of inflammatory cells into the CNS (11). These data indicated that Fas and its ligand are important for the progression of clinical signs of EAE. We hypothesized that $FasL^+$ myelin-reactive lymphocytes contributed to the pathogenesis of EAE by lysing Fas-expressing targets in the CNS. However, our active induction experiments could not distinguish FasL expressed on activated, myelin-specific $CD4^+$ T cells from that expressed on other inflammatory or resident CNS cells. Furthermore, because the mitigation of EAE was the dominant effect of the *lpr* and *gld* mutations, we were unable to determine whether a Fas-mediated lytic interaction might also be contributing to the recovery from actively induced disease.

Therefore, to further examine the role of Fas and its ligand in EAE, we conducted adoptive transfer experiments of myelin-reactive wild-type, *lpr*, or *gld* lymphocytes into congenic wild-type, *lpr*, and *gld* hosts (H-2^u). In our initial experiments, conventional (polyclonal) MBP-specific lymphocytes isolated from primed animals and cultured in MBP for 4 d were used as donor cells. Both MBP-specific cells and pertussis toxin were required for recipient animals to develop EAE. To complete the reciprocal transfers, it was necessary to introduce the mutations (especially *lpr*) onto an MBP-specific TCR transgenic mouse. TCR transgenic wild-type, *lpr*, and *gld* cells from unimmunized mice responded equally well to in vitro stimulation with MBP, and more vigorously compared with the nontransgenic cells (data not shown). These cultures contained 62–78% $CD4^+$ and 2–5% $CD8^+$ T lymphocytes, 12–18% B cells, and <5% macrophages.

Fas-mediated Lysis Is Important in the Initiation/Early Progression of EAE

Host-derived Fas Contributes to the Pathogenesis of EAE. In our initial experiments, conventional MBP-specific wild-type (B10.PL) cells were adoptively transferred into congenic wild-type, *lpr*, and *gld* hosts (Fig. 1 and Table I). Clinical signs of disease consistently appeared 20–30 d after transfer in recipients of all three genotypes. 88% of the wild-type animals developed clinical signs of EAE, with a peak disease severity corresponding to partial hindlimb paralysis (3.0 ± 0.91; median = 3). On the other hand, *lpr* recipients were quite resistant to the development of EAE, with only 12% of the animals exhibiting clinical disease. The two *lpr* mice that did develop clinical signs of EAE developed very mild disease corresponding to tail paralysis (grades 1 and 2; $P = 0.032$ versus wild-type). These data are consistent with our previous study in which the *lpr* mutation mitigated the development of actively induced EAE. In contrast to our previous study using an active induction paradigm, *gld* recipients of adoptively transferred wild-type lymphocytes were overall highly susceptible to disease, with an incidence of 75% and a mean peak disease severity similar to that observed in wild-type recipients (3.0 ± 1.1; median = 2.25). However, unlike in the wild-type recipients, in which all animals developing clinical signs of EAE progressed to clinical scores of 2–4, the severity of EAE in *gld* animals was highly variable. Some (9 out of 28) *gld* recipients developed only mild clinical signs of EAE (grade 0–1.5), similar to that observed in the *lpr* recipients. How-

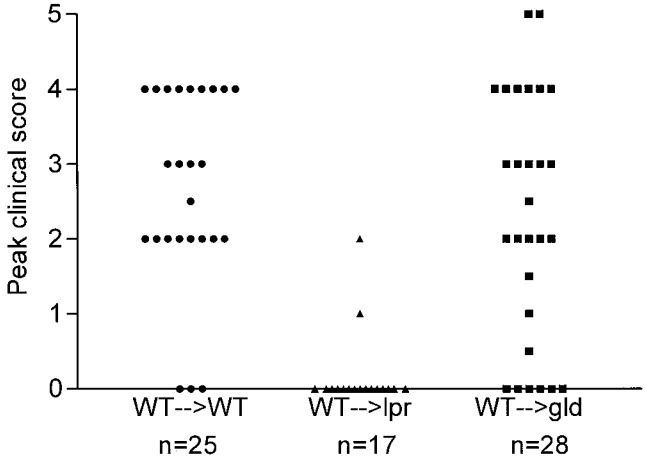

Figure 1. Peak clinical scores of EAE adoptively transferred with wild-type lymphocytes vary with recipient genotype. 5–8 × 10^7 conventional (i.e., nontransgenic) wild-type lymphocytes were injected intravenously into sublethally irradiated (350 rads) B10.PL (WT) or congenic *lpr* and *gld* recipients along with 200 ng pertussis toxin. Animals were given two additional injections of pertussis toxin on days 3 and 7. Clinical signs were monitored daily and graded on a scale from 0 to 5 as described in Materials and Methods. Data were pooled from four experiments.

Table I. *Adoptive Transfers of Nontransgenic Wild-type or gld Lymphocytes into B10.PL and Congenic lpr and gld Mice**

Recipient genotype	Incidence of clinical signs (%)	Mean peak clinical score	Mean peak disease severity
Wild-type donor cells			
B10.PL	22/25 (88)	2.3 ± 1.5	3.0 ± 0.91
lpr/lpr	2/17 (12)	0.16 ± 0.50‡	1.5 ± 0.71‡
gld/gld	21/28 (75)	2.0 ± 1.7	3.0 ± 1.1
gld donor cells			
B10.PL	4/18 (22)	0.36 ± 0.72§	1.6 ± 0.48§
lpr/lpr	0/11 (0)	0	NA
gld/gld	4/15 (27)	0.90 ± 1.6‖	3.4 ± 0.75¶

*5–8 × 10^7 wild-type or *gld* lymphocytes were injected intravenously into sublethally irradiated (350 rads) B10.PL or congenic *lpr* and *gld* recipients along with 200 ng pertussis toxin. Animals were given two additional injections of pertussis toxin on days 3 and 7. Clinical signs were monitored daily and graded on a scale from 0 to 5 as described in Materials and Methods. Data were pooled from two (*gld* donors) or four (wild-type donors) experiments.
‡Mean peak clinical score, $P = 4.81 \times 10^{-7}$; mean peak disease severity (only includes animals with grade 1 or higher), $P = 0.032$ (versus B10.PL).
§Mean peak clinical score, $P = 9.24 \times 10^{-6}$; mean peak disease severity $P = 0.0066$ (versus B10.PL recipients of wild-type cells).
‖$P = 0.038$ versus *gld/gld* recipients of wild-type cells.
¶$P = 0.0077$ versus B10.PL.

ever, most (17 out of 28) *gld* recipients exhibited clinical scores of grades 2 to 4, and two mice progressed to the most severe stage of disease (grade 5).

The adoptive transfer of MBP-specific TCR transgenic wild-type lymphocytes into wild-type and congenic *lpr* and *gld* hosts confirmed the results obtained when conventional wild-type lymphocytes were transferred (Fig. 2 A and Table II). *lpr* recipients were resistant to the development of clinical signs of EAE transferred by transgenic wild-type cells. In contrast, all of the wild-type and *gld* animals developed clinical signs of EAE ~2 wk after cell transfer, with peak clinical scores corresponding to moderate hindlimb weakness (wild-type, 2.1 ± 0.97, median = 2; *gld*, 2.4 ± 0.78, median = 2). Thus, the adoptive transfers of myelin-reactive wild-type T cells demonstrate that Fas expressed within the host (presumably in the CNS) plays an important role in the pathogenesis of EAE.

Adoptively Transferred Lymphocytes Are Not Eliminated in lpr Recipients. Having established that *lpr* recipients develop only mild clinical signs of adoptively transferred EAE, we asked whether this amelioration of disease was actually due to a paucity of Fas^+ targets in the CNS, or if it was simply because the *lpr* host lymphocytes, which are reported to express elevated levels of FasL (21), were eliminating the activated, Fas^+ lymphocytes we transferred. We were unable to unequivocally identify the donor lymphocytes in adoptive transfer experiments described above.

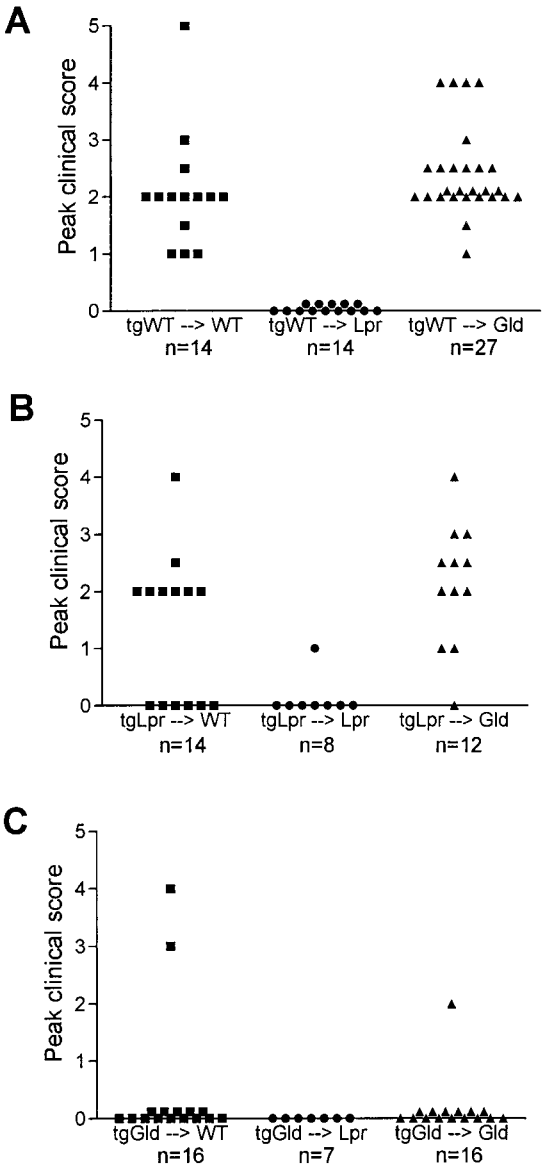

Figure 2. Reciprocal adoptive transfers of MBP-specific TCR transgenic wild-type, *lpr*, or *gld* lymphocytes into B10.PL or congenic *lpr* and *gld* mice. 10^7 activated, transgenic wild-type (A), *lpr* (B), or *gld* (C) lymphocytes were injected intravenously into sublethally irradiated (450 rads) B10.PL or congenic *lpr* and *gld* recipients. Clinical signs were monitored daily and graded on a scale from 0 to 5 as described in Materials and Methods. Data were pooled from two separate experiments for each type of donor cell.

Therefore, 5 × 10^7 B6 anti-B10.PL cells (H-2^b) from a 5-d MLR were injected into sublethally irradiated wild-type or *lpr* hosts (H-2^u). Spleens were removed at various days after transfer and stained for L^d, a marker of the H-2^u haplotype, and K^b, a marker of the donor cells. Fig. 3 shows spleen sections removed from wild-type and *lpr* mice 3 d after transfer. Comparable numbers of K^b-expressing cells were detected in the T cell zones, but not follicles, of both wild-type and *lpr* animals given donor cells. Spleens taken from wild-type and *lpr* animals that were irradiated but did not receive K^b-expressing cells showed staining for L^d, but not

Table II. *Adoptive Transfers of MBP-specific TCR Transgenic Wild-type, lpr, or gld Lymphocytes in B10.PL and Congenic lpr and gld Mice**

Recipient genotype	Incidence of clinical signs (%)	Mean peak clinical score	Mean peak disease severity
Wild-type donor cells			
B10.PL	14/14 (100)	2.1 ± 0.97	2.1 ± 0.97
lpr/lpr	0/14 (0)	0 ± 0‡	–
gld/gld	27/27 (100)	2.4 ± 0.78	2.4 ± 0.78
lpr donor cells			
B10.PL	8/14 (57)	1.3 ± 1.3	2.3 ± 0.70
lpr/lpr	1/8 (12)	0.12 ± 0.35§	1
gld/gld	11/12 (92)	2.1 ± 1.1	2.3 ± 0.87
gld donor cells			
B10.PL	2/16 (12)	0.44 ± 1.2‖	3.5 ± 0.71
lpr/lpr	0/7 (0)	0 ± 0	–
gld/gld	1/16 (6)	0.13 ± 0.52¶	2

*10^7 activated, transgenic wild-type, *lpr*, or *gld* lymphocytes were injected intravenously into sublethally irradiated (450 rads) B10.PL or congenic *lpr* and *gld* recipients. Clinical signs were monitored daily and graded on a scale from 0 to 5 as described in Materials and Methods. Data were pooled from two separate experiments for each type of donor cell.
‡$P = 9.4 \times 10^{-9}$ versus B10.PL.
§$P = 0.020$ versus B10.PL.
‖$P = 0.00023$ versus B10.PL recipients of transgenic wild-type cells.
¶$P = 4.2 \times 10^{-13}$ versus *gld/gld* recipients of wild-type cells.

K^b (data not shown). 5 d after transfer, donor cells were reduced to a similar degree in spleens of both wild-type and *lpr* recipients compared with day 3 levels (data not shown).

Lpr recipients of conventional, nontransgenic lymphocytes were monitored for clinical signs of disease for the duration of each adoptive transfer experiment and therefore were not examined for histological signs of EAE. However, the fact that some *lpr* hosts did develop clinical signs of EAE suggests that the mutation does not preclude the infiltration of cells into the CNS. *Lpr* recipients of MBP-specific TCR transgenic wild-type lymphocytes (see below) were examined histologically at 9 and 17 d after cell transfer and found to have small infiltrates (grade 0.5–1) in the CNS (data not shown). This is consistent with our previous study in which we found Fas was not required for lymphocytes to infiltrate the CNS after active induction of EAE (11). These data indicate that the *lpr* hosts are not abnormally eliminating the adoptively transferred lymphocytes. Thus, the lack of EAE we have observed in *lpr* recipients is probably due to a role for host-derived Fas that is important for the progression of severe disease.

Fas-deficient (lpr) MBP-specific TCR Transgenic Lymphocytes Transfer EAE to Wild-type and gld, but Not lpr Recipients. The adoptive transfer of nontransgenic *lpr* lymphocytes led to the development of a lethal graft-versus-host disease (GVHD) in many of the wild-type recipient animals before the onset of clinical signs of EAE (data not shown). We reasoned that skewing the T cell repertoire to a predominantly MBP-specific $CD4^+$ T cell population would prevent the transfer of GVHD in our adoptive transfer experiments, and thus introduced the *lpr* mutation onto an MBP-specific TCR transgenic mouse. Wild-type and *gld* recipients of transgenic *lpr* lymphocytes did not develop GVHD, but did develop clinical signs of EAE ~2 wk after cell transfer. The incidence of clinical disease induced by *lpr* donor cells was lower (8 out of 14; 57%) than that induced by wild-type lymphocytes (14 out of 14; 100%) after transfer into wild-type recipients (Fig. 2 B and Table II). In those wild-type recipients of transgenic *lpr* cells that developed EAE, the peak disease severity corresponded to moderate hindlimb weakness (2.3 ± 0.70; median = 2). *Gld* recipients were more susceptible than wild-type hosts to disease induced by transgenic *lpr* cells, with 92% developing clinical signs of EAE. The severity of EAE induced by the transfer of transgenic *lpr* cells into *gld* recipients was highly variable. The mean peak disease severity of *gld* recipients was 2.3 ± 0.87 (median = 2).

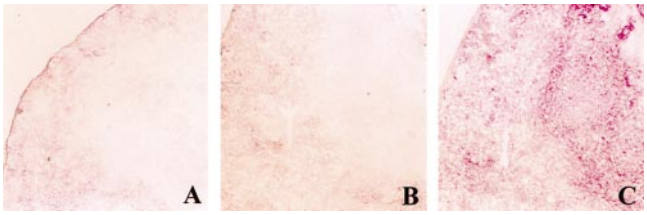

Figure 3. Adoptively transferred lymphocytes are not eliminated by *lpr* recipients. B10.PL (A) or B10.PL.*Fas^{lpr}* (B and C) mice were sublethally irradiated. 5×10^7 B6 anti-B10.PL cells from a 5-d MLR were injected intravenously into some animals (A–C), with the remaining mice serving as negative controls (data not shown). On day 3 after transfer, spleens were removed and sections were stained for L^d (C) and K^b (A-B) to detect host tissue and donor cells, respectively. Peroxidase reaction was detected with VIP substrate, resulting in a pinkish/purple signal. No counterstain was used. Region shown in B corresponds to that in C. Original magnification: ×131.

We also examined the induction and progression of adoptively transferred EAE in the complete absence of Fas by transferring Fas-deficient (*lpr*) MBP-specific TCR transgenic lymphocytes into *lpr* mice (Fig. 2 B and Table II). The incidence of disease transferred by transgenic *lpr* donor cells into *lpr* recipients was greatly reduced compared with the incidence observed in wild-type and *gld* recipients, with only 12% (1 out of 8) of *lpr* recipient mice developing EAE of an average peak clinical score of 0.12 ± 0.35 ($P = 0.020$ versus wild-type; $P = 7.96 \times 10^{-5}$ versus *gld*). The onset of disease in the one *lpr* recipient that developed clinical signs of EAE occurred 24 d after cell transfer, which was slightly delayed compared with disease onset in wild-type and *gld* recipients of wild-type or *lpr* lymphocytes, and disease was mild (grade 1). Thus, Fas-deficient transgenic *lpr* lymphocytes were able to transfer EAE into wild-type and *gld*, but not *lpr* recipients. Furthermore, the disease that developed after the transfer of *lpr* cells was similar in onset and severity to that observed when wild-type lymphocytes were transferred.

In these experiments, the transgenic *lpr* lymphocytes transferred could not have been eliminated by $FasL^+$ cells in the *lpr* recipients. Therefore it is highly unlikely that the lack of EAE we have observed in the *lpr* recipient mice is due to rejection of the donor lymphocytes. Instead, these data demonstrate that Fas expressed by the recipient animals (presumably in the CNS) is an important determinant for the pathogenesis of EAE.

FasL Expressed on Donor Lymphocytes Contributes to the Development of EAE. To determine the relative role of FasL expressed by MBP-reactive T lymphocytes in the initiation of EAE, conventional MBP-specific lymphocytes from *gld* mice were adoptively transferred into congenic wild-type, *lpr*, and *gld* recipients. The incidence of disease transferred with MBP-reactive *gld* lymphocytes was rather low, with only 22% of wild-type and 27% of *gld* animals developing clinical signs of EAE (Table I). In the few wild-type and *gld* recipients that exhibited clinical signs of EAE, the peak disease severity corresponded to complete tail paralysis (grades 1, 1.5, 2, 2) and partial to complete hindlimb paralysis (grades 2.5, 3.5, 4, 4) for wild-type and *gld* recipients, respectively. None of the 11 *lpr* mice that received *gld* lymphocytes developed clinical signs of disease over the duration of the experiments. These data suggest that FasL expressed on the donor lymphocyte population is important for the initiation of the inflammatory cascade of events leading to development of EAE. The apparent exacerbation of clinical disease in the four *gld* recipients may reflect an inability of those animals to control the expansion of inflammatory cells (see below) that have initiated the disease, presumably through a Fas/FasL-independent mechanism.

Similar results were obtained when MBP-specific TCR transgenic *gld* lymphocytes were transferred into wild-type B10.PL and congenic *lpr* and *gld* hosts (Fig. 2 C and Table II). None of the seven *lpr* recipients developed clinical signs of EAE. The onset of disease in wild-type and *gld* recipients of transgenic *gld* lymphocytes was slightly delayed (20 ± 6), but did not statistically differ from that seen in animals receiving FasL-expressing cells. The incidence of disease transferred with *gld* lymphocytes was low compared with that transferred by transgenic wild-type lymphocytes. Only 12% of wild-type and 6% of *gld* hosts developed EAE with average peak clinical scores of 0.44 ± 1.2 and 0.13 ± 0.52, respectively. Thus, the adoptive transfer of MBP-specific TCR transgenic *gld* T cells further substantiates our conclusion that FasL expressed on donor lymphocytes is important for the initiation and/or progression of EAE.

Fas-dependent Lysis Is also Involved in the Remission of EAE

Host-derived FasL Is Important in Recovery from EAE. While analyzing the results from the transfer of conventional wild-type lymphocytes into the various hosts, we noticed that the duration of the clinical signs of EAE after the onset of disease varied by genotype of the recipient. Wild-type recipients recovered from the acute phase of EAE, regardless of the severity of disease that had developed, with most entering complete remission within 30 d of the onset of clinical signs (average $= 26 \pm 12$ d) (Figs. 4 A and 5 A). *Lpr* recipients also recovered from acute EAE, although the duration of disease (average $= 5 \pm 3$ d) was much shorter compared with that of wild-type recipients and correlated with their mild disease severity rather than their genotype (Fig. 4 C and data not shown). Surprisingly, *gld* recipients fell into two groups (Figs. 4 B and 5 B). Those *gld* mice that developed mild disease (grade ≤ 2) were able to rapidly recover from the acute phase of EAE (average $= 20 \pm 13$ d compared with 31 ± 13 d for wild-type recipients [grade ≤ 2]). In contrast, all *gld* recipients that reached a clinical score greater than grade 2 developed a more chronic disease, exhibiting clinical signs of EAE for well over 35 d (average $= 50 \pm 14$ d compared with 22 ± 11 d for wild-type recipients greater than grade 2; $P = 2.4 \times 10^{-5}$). These data suggest that there is a source of FasL in the recipient that is lacking in the *gld* mice and is important in the recovery from severe disease.

A relationship between the severity of disease and duration of clinical signs of EAE in *gld* recipients similar to that observed after transfer of conventional wild-type donor

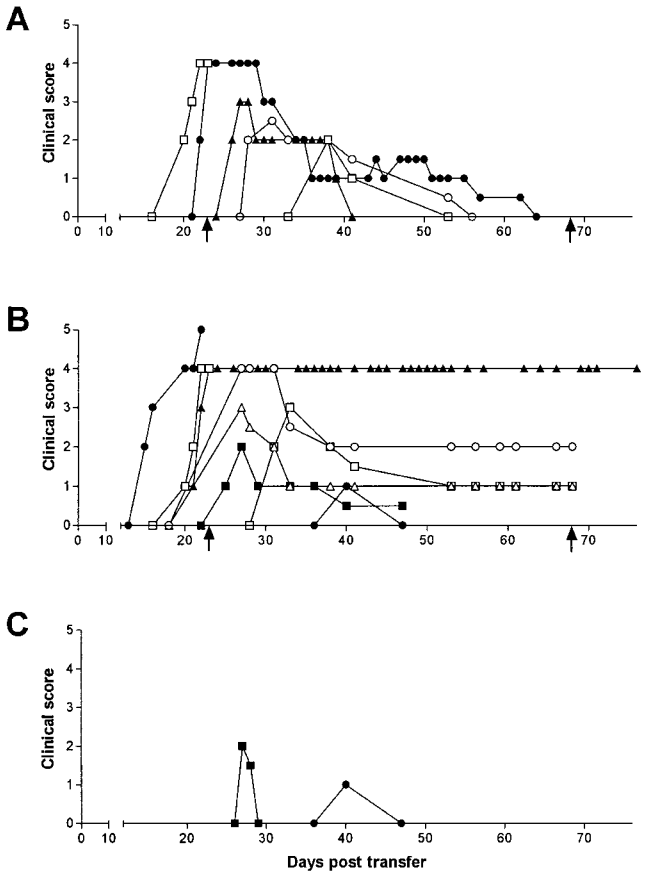

Figure 4. Daily clinical scores of EAE for wild-type, *lpr*, and *gld* recipients of nontransgenic wild-type lymphocytes. EAE was induced as described in Fig. 1. The daily clinical scores for representative wild-type (A), *lpr* (C), and *gld* (B) recipient mice are shown. Animals represented with open symbols were killed 23 or 68 d after transfer (arrows) for histological analyses shown in Fig. 6.

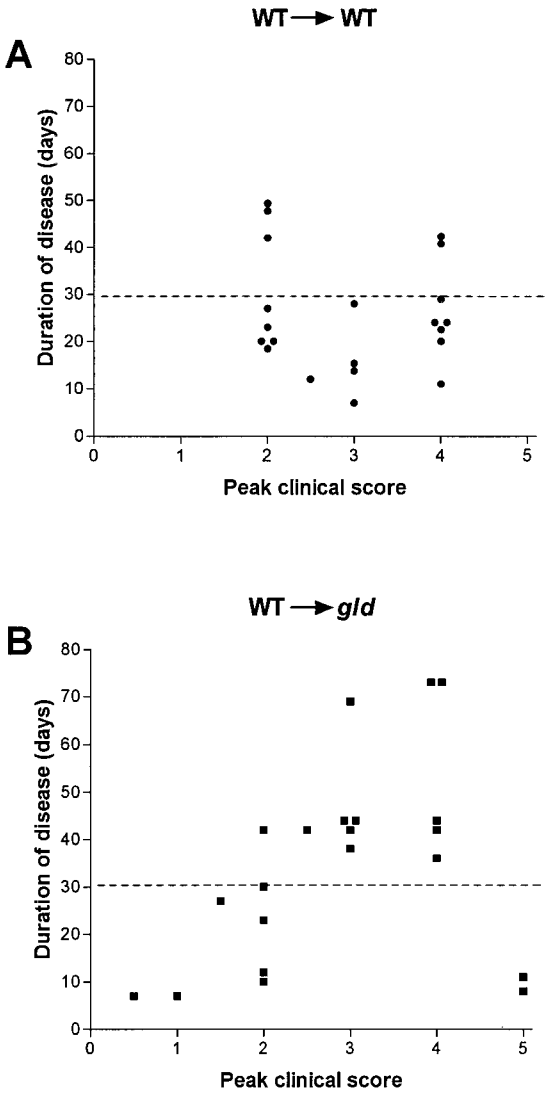

Figure 5. Duration of clinical signs of adoptively transferred EAE is prolonged in *gld* recipients. EAE was induced as described in Fig. 1. The duration of clinical signs of EAE after the onset of disease versus the peak clinical score for wild-type (A) and *gld* (B) recipients is shown. All *lpr* and 18 out of 21 wild-type recipients recovered from the acute phase of EAE before the termination of the experiments at 90 d after cell transfer. In contrast, 13 out of 21 *gld* recipients (62%) still exhibited clinical signs of EAE on day 90 ($P = 0.00098$). Animals killed for histological analyses or for ethical reasons were not included in these analyses.

cells was also evident when disease was induced with TCR transgenic cells. 82% (9 out of 11) of the wild-type recipients of transgenic wild-type cells recovered from the acute phase of EAE within 15 d of the onset of clinical signs, with the remaining 2 animals entering complete remission within 20 d of clinical onset (average = 12 ± 5 d). In contrast, 57% (12 out of 21) of the *gld* recipients of wild-type transgenic cells, and all of those exceeding a clinical score of grade 2, exhibited clinical signs of EAE for >15 d (average = 18 d ± 10; $P = 0.040$). Likewise, when TCR transgenic *lpr* cells were transferred, only 2 out of 8 wild-type recipients remained clinically affected for 15 d or more (durations were 16 and 23 d). In contrast, 45% of *gld* recipients (5 out of 11) exhibited clinical signs of EAE for 15 d or more (durations were 15, 23, 30, 45, and 46 d, with the latter two still affected at termination of experiment 56 d after cell transfer). Thus, host-derived FasL can function in the regulation of ongoing disease, possibly by eliminating the infiltrating Fas^+ T cells.

Fas-dependent Death of T Lymphocytes May Be Involved in Recovery from EAE. The experiments above indicated that severely affected *gld* recipients were unable to recover from the acute phase of EAE as well as their wild-type counterparts. In an attempt to understand the differences between acute and chronic disease, we decided to compare CNS lesions in wild-type and *gld* recipients of conventional wild-type lymphocytes at both early/acute (day 23 after cell transfer) and late/chronic (day 68 after cell transfer) stages of disease (Fig. 6). Comparable levels of inflammation (grade 4) were found in the lower spinal cords of a wild-type and *gld* recipient during the acute phase of disease (Fig. 6, A and B).

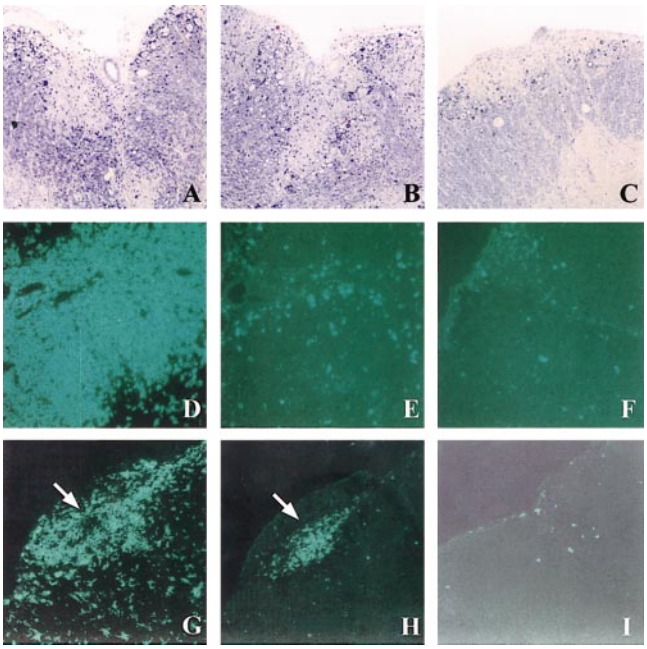

Figure 6. Infiltration of the CNS during acute and chronic stages of EAE in wild-type and *gld* hosts. EAE was induced as described in Fig. 1. 23 (A, B, D–F) and 68 (C and G–I) d after cell transfer, wild-type (A, F, I) and *gld* (B–E, G, and H) animals were killed for histological and immunohistochemical analyses. (A–C) Anterior columns of lower spinal cord sections stained with toluidine blue. A, wild-type mouse 23 d after transfer; clinical score = 4, inflammation score = 4. B, *gld* mouse 23 d after transfer; clinical score = 4, inflammation score = 4. C, *gld* mouse 68 d after transfer; clinical score = 1, inflammation score = 2. (D and G) Lower spinal cord sections immunostained with anti-CD11b (macrophage/microglia marker; green). D, *gld* mouse 23 d after transfer; clinical score = 4. G, *gld* mouse 68 d after transfer; clinical score = 2. (E, F, H, and I) Lower spinal cord sections immunostained with anti-CD4 (green). E, *gld* mouse 23 d after transfer; clinical score = 4. The region shown in E corresponds to that in D. F, wild-type mouse 23 d after transfer; clinical score = 4. H, *gld* mouse 68 d after transfer; clinical score = 2. The region shown in H corresponds to that in G. I, wild-type mouse 68 d after transfer; clinical score = 0. Original magnification: A–C, G, H, and I, ×131; D, E, and F, ×262.

The infiltrates were comprised primarily of $CD11b^+$ cells, many of which were also TUNEL positive (Fig. 6 D and data not shown). Roughly equal numbers of $CD4^+$ cells were detected in inflammatory lesions from acutely affected wild-type and *gld* mice, with ~4% of them undergoing apoptotic cell death (i.e., TUNEL positive; Fig. 6, E and F, and data not shown). The only potential difference observed between wild-type and *gld* lesions at this stage of disease was a more diffuse infiltration, especially of $CD4^+$ cells, in the *gld* recipient (compare Fig. 6, A and F with B and E).

68 d after transfer, inflammation scores had diminished somewhat in the wild-type animals that had clinically recovered from EAE and in the *gld* recipients that still exhibited clinical signs of disease (wild-type, grade 1–2; *gld*, grade 1.5–2.5) (Fig. 6 C and data not shown). The degree of apoptotic cell death was greatly reduced in both wild-type and *gld* lesions compared with that seen at the acute stage of disease (data not shown). Wild-type lesions were comprised of fewer $CD11b^+$ cells that had a distribution similar to that observed in acute lesions (data not shown). A few, scattered $CD4^+$ cells were also present in wild-type lesions (Fig. 6 I). In contrast, clusters or "nests" of $CD4^+$ cells were observed in many of the lesions in *gld* spinal cords (Fig. 6 H), and were often surrounded by $CD11b^+$ cells (Fig. 6 G). The correlation between the presence of these T cell nests and recipient genotype was not absolute, in that $CD4^+$ cell clusters were not found in all spinal cord sections from *gld* recipients and small nests were observed in some sections of wild-type spinal cord. However, there was a direct correlation between the severity (and possibly the duration) of chronic disease in affected recipient animals, regardless of their genotype, and the average number of $CD4^+$ T cell nests (Table III). Taken in conjunction with results from our adoptive transfer experiments, we believe that the presence of many $CD4^+$ cells in *gld* recipients is indicative of an impaired ability of these FasL-deficient animals to curtail expansion of activated, Fas^+ lymphocytes. A combined immunohistochemical and TUNEL analysis of actively induced EAE lesions from wild-type and *lpr* mice has also implicated a Fas-mediated lytic mechanism for the elimination of $CD4^+$ and $CD8^+$ T lymphocytes (Sabelko-Downes, K.A., A.H. Cross, and J.H. Russell, manuscript in preparation).

Discussion

The data presented here provide conclusive evidence that a Fas-mediated lytic mechanism is involved in the pathogenesis of EAE, as FasL-deficient lymphocytes transfer attenuated disease, whereas the absence of Fas in recipient animals dramatically attenuated the development of clinical signs of EAE induced by encephalitogenic, FasL-expressing lymphocytes. The pathogenic, Fas-dependent lytic interaction presumably occurs between $FasL^+$ lymphocytes and Fas-expressing targets in the CNS. FasL-deficient *gld* lymphocytes could transfer EAE to some *gld* mice, and an *lpr* recipient did develop clinical disease. This, along with our previous study in which EAE was actively induced in some *lpr* and *gld* mice (11), demonstrates that although a Fas-dependent lytic mechanism is important, the pathogenesis of EAE is not solely dependent upon Fas.

The reported role for Fas in diabetes (22, 23) has recently been questioned, as the adoptive transfer of diabetogenic lymphocytes into NOD.*lpr* mice resulted in a partial rejection of the donor cell population (24). The authors suggested that an interaction between $FasL^+$ recipient cells and Fas^+ donor lymphocytes contributed to donor cell rejection by the NOD.*lpr* mice. We believe that this process only poses a potential problem when the *lpr* allele is present in animals whose genetic background predisposes them to develop accelerated lymphoproliferative and autoimmune disease. C57BL/6 and B10.PL mice do not fall into this

Table III. *Nests of $CD4^+$ T Cells Are Predominantly Found in Spinal Cord Sections from Chronically Affected gld Recipients of Conventional Wild-type T Cells**

Recipient genotype	Peak clinical score	Clinical score at time of death	Duration of clinical signs of EAE (days)‡	Mean number of nests per spinal cord cross-section§
B10.PL	2	0	19	0
B10.PL	2	0	27	0.75 ± 0.96
gld	4	4	73‖	2.8 ± 1.5
gld	4	2	73‖	2.8 ± 1.9
gld	4	2	43‖	1.6 ± 2.1
gld	3	1	43‖	1 ± 1.7
gld	2	0	30	0

*EAE was induced as described in Fig. 1.
‡All animals were killed at 67 or 68 d after cell transfer, except *gld/gld* mice exhibiting clinical signs of disease for 73 d, which were killed at 94 d after cell transfer.
§Nests or clusters of >10 immediately adjacent $CD4^+$ cells were counted in 4–5 representative lower spinal cord cross-sections per mouse. The average number of clusters per cross-section ± SD is shown for each animal.
‖Animals were still sick at termination of experiment.

category, as the severe lymphoproliferative disease associated with the *lpr* mutation does not develop until animals are ~12–16 wk old (Sabelko-Downes, K.A. and J.H. Russell, unpublished observation). B10.PL and congenic *lpr* and *gld* donor and recipient animals used in all of our experiments were 6–8 wk old at the time of transfer. Thus, donor lymphocytes were transferred into recipient mice at least 4–6 wk before the onset of severe lymphoproliferative disease. This issue was also addressed by the experiment in which we detected comparable levels of donor cells in the spleens of wild-type and *lpr* recipients 3 and 5 d after cell transfer (Fig. 3).

Two additional experiments substantiate a role for Fas in the pathogenesis of EAE. First, we (11) and others (12, 13) have previously observed a difference in the severity of actively induced EAE between young (6–8 wk old) wild-type and *lpr* mice on the B10.PL and C57BL/6 backgrounds, respectively. Furthermore, in our study we were able to transfer Fas-deficient lymphocytes into wild-type or *lpr* mice and again we found that the absence of Fas in the host mitigated the clinical signs of EAE (Fig. 2 B and Table II). In this transfer the donor *lpr* lymphocytes could not have been eliminated by $FasL^+$ cells in the *lpr* recipients. It is highly unlikely that the lack of EAE we have observed in *lpr* recipients results from rejection of the donor lymphocyte population. Thus, the data in this paper support our conclusion that a Fas-dependent mechanism is important for the pathogenesis of EAE.

Our adoptive transfer experiments have also revealed a role for a Fas-dependent lytic interaction in the recovery from disease, as most FasL-deficient *gld* recipients developed chronic EAE. This Fas-dependent regulatory mechanism apparently involves targets, and possibly effectors, that are distinct from those involved in the Fas-mediated lytic mechanism that helps to initiate EAE. The FasL-expressing effector cells are host-derived, and could be resident CNS cells or cells recruited into the CNS during inflammation, whereas the targets are likely to be activated, Fas^+ lymphocytes infiltrating the CNS. Wild-type recipients of transgenic *lpr* lymphocytes did not develop prolonged signs of EAE. This suggests that a population of activated lymphocytes that are targets of Fas-mediated lysis during the recovery from EAE may be host-derived cells recruited into the CNS during the course of disease. In *gld* recipients, these Fas^+ host-derived cells and any donor *lpr* lymphocytes present in the CNS could help establish chronic EAE.

We have found that Fas-deficient $CD4^+$ lymphocytes were able to invade the CNS and that severe inflammation developed in the CNS of FasL-expressing wild-type hosts. This indicates that FasL does not provide a primary defense against infiltration of activated T cells (i.e., immune privilege). However, our adoptive transfer experiments suggest that once FasL is induced on cells recruited into or residing within the CNS by inflammation, it may function in a mechanism analogous to that invoked for immune privilege (25, 26) to limit the expansion of activated T cell populations in the CNS. In *gld* recipients, infiltration of the CNS does not induce functional FasL, and consequently lymphocytes can accumulate and continue to damage the CNS. Although Fas-dependent T cell suicide could also occur, we were unable to ascertain the relative contribution of this autonomous lytic interaction to the elimination of the infiltrating T cell population from the experiments described here. Nevertheless, any potential treatment for MS designed to disrupt Fas-mediated lytic interactions must take into consideration this dual role for FasL in EAE.

This paper provides in vivo functional evidence implicating both donor lymphocytes and host-derived elements as $FasL^+$ effectors that contribute to the progression of EAE. It further suggests the host CNS is a major source of Fas-expressing targets. Although this study has not determined the specific cell types that serve as targets and effectors of Fas-mediated lytic interactions in EAE, we can speculate as to their identity. It is likely that oligodendrocytes are the Fas^+ targets of myelin disruption in EAE, as they have been identified as Fas-expressing cells in MS lesions (6–8) and are able to express Fas and serve as lytic targets in vitro (6). FasL has been detected on glial cells (7), and in particular microglia (6, 8), in MS lesions, making these resident cells of the CNS candidate effectors of Fas-dependent lysis. Macrophages recruited into the CNS during EAE are also potential effectors of Fas-mediated death.

In sum, these experiments have described both a novel pathogenic mechanism and a regulatory process of T cell–mediated autoimmune disease in the CNS, each mediated by an interaction between Fas and its ligand. We propose the following model to describe the dual role of Fas-dependent lytic interactions in the initiation of and recovery from EAE. After an initial stimulation, the activated and differentiated T cell enters the CNS, encounters its antigen presented by a resident APC (e.g., endothelial cells, microglia, astrocytes) and is activated for the second time. At this point, the Th1 cell can function as an effector and/or target of Fas-dependent lysis. The effector T cell also secretes proinflammatory cytokines, including IFN-γ and LT/TNF-α, which can enhance expression of MHC molecules and promote cytokine/chemokine production by microglia and astrocytes (27–29). IFN-γ and TNF-α can also induce expression of Fas (30–32) or FasL (33, 34) on various cell types. Consequently, they may contribute to the pathogenesis of EAE by augmenting expression of this lytic receptor on oligodendrocytes, for example, and/or by inducing expression of its ligand on microglia or astrocytes. Although the relative contributions of the effector cell populations is not clear, it is likely that FasL expressed by infiltrating lymphocytes plays a primary role in the initiation of disease, as it seems the FasL expressed on cells residing within or recruited into the CNS must be induced by products of the inflammatory cascade. However, upon induction, FasL expressed on cells present in the CNS may function in the pathogenic process, and certainly becomes a major participant in the recovery from EAE. The balance achieved between the Fas-mediated death of CNS cell targets, which would enhance the progression of EAE, and the Fas-dependent death of infiltrating T cells, which would contribute to remission of disease, will in part determine the clinical manifestations of EAE.

The authors are grateful to Brian Rush for technical assistance with the adoptive transfer experiments and Manuel San for technical assistance with the histological analysis.

This work was supported in part by grants from the MS Society (RG2835) and the National Cancer Institute (CA28533).

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Received for publication 21 December 1998 and in revised form 12 February 1999.

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