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Expression of the Long Form of Human FLIP by Retroviral Gene Transfer of Hemopoietic Stem Cells Exacerbates Experimental Autoimmune Encephalomyelitis

Mounira Djerbi,2,* Khairul-Bariah Abdul-Majid,† Manuchehr Abedi-Valugerdi,* Tomas Olsson,† Robert A. Harris,† and Alf Grandien*

Subsidence of inflammation and clinical recovery in experimental autoimmune encephalomyelitis (EAE) is postulated to involve apoptosis of inflammatory cells. To test this concept, we examined the effects of overexpressing the long form of human FLICE-inhibitory protein, a potent inhibitor of death receptor-mediated apoptosis, in myelin oligodendrocyte glycoprotein-induced EAE in DBA/1 mice. We found that overexpression of the long form of human FLICE-inhibitory protein by retroviral gene transfer of hemopoietic stem cells led to a clinically more severe EAE in these mice compared with control mice receiving the retroviral vector alone. The exacerbated disease was evident by an enhanced and prolonged inflammatory reaction in the CNS of these animals compared with control mice. The acute phase of EAE was characterized by a massive infiltration of macrophages and granulocytes and a simultaneous increase in TNF-α production in the CNS. In the chronic phase of the disease, there was a prolonged inflammatory response in the form of persistent CD4+ T and B cells in the CNS and a peripheral Th1 cytokine bias caused by elevated levels of IFN-γ and reduced levels of IL-4 in the spleen. Our findings demonstrate that death receptor-mediated apoptosis can be important in the pathogenesis of EAE and further emphasize the need for effective apoptotic elimination of inflammatory cells to achieve disease remission. The Journal of Immunology, 2003, 170: 2064–2073.

Expression of the Long Form of Human FLIP by Retroviral Gene Transfer of Hemopoietic Stem Cells Exacerbates Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE)1 is an inflammatory disease of the CNS and is an animal model for the human disease multiple sclerosis (MS). Chronic relapsing or nonrelapsing forms of EAE can be induced in rodents by active immunization with myelin Ags such as myelin oligodendrocyte glycoprotein (MOG) (1) or by passive transfer of MOG-specific CD4+ or CD8+ T cells and anti-MOG Abs (2, 3). The pathology of EAE is well described, differs between models, but is generally characterized by increased permeability of the blood-brain barrier, perivascular inflammatory infiltrates comprised of T cells, B cells, macrophages, and granulocytes, and demyelination leading to an ascending paralysis of the extremities. The principal targets of the autoimmune attack are oligodendrocytes and myelin. However, the precise molecular mechanisms causing myelin disruption and damage to axons in the CNS are still unclear. It is generally considered that CD4+ T cells initiate EAE (4), and Th1-associated proinflammatory cytokines such as IFN-γ and TNF-α are important disease-promoting cytokines (5). We have recently described a pertussis toxin–induced MOG-induced EAE model in DBA/1 mice that shares the salient pathological features of human MS, namely involvement of both CD4+ and CD8+ T cells, B cells (6), and FcyR (7), and that is characterized by multiple demyelinated lesions in the CNS (8).

Spontaneous recovery and/or prevention from EAE are in general associated with a deviation from Th1 to Th2 cytokine responses (9, 10); however, there are contradictory results as to the protective role of Th2 cytokines (11, 12), and Th1 cytokines have also been reported to be critical for inhibition of EAE (13, 14). Suppression of autoimmune CD4+ T cells by regulatory cells may be another component of the disease-limiting process (15–19). Furthermore, deletion of encephalitogenic T cells through activation-induced cell death (AICD) has also been proposed to contribute to the resolution of inflammation (20–22).

AICD through Fas/Fas ligand (FasL) interactions is an important mechanism for regulation of immune responses, as exemplified by the abrogated in vitro Ag-stimulated suicide of activated mature T cells from lpr and gld mice that carry mutations in the Fas and Fasl genes, respectively, and by the impaired deletion of Vβ8+ T cells in staphylococcal enterotoxin B-treated lpr mice (23–25). Moreover, the systemic autoimmune manifestations in these animals indicate that Fas-mediated AICD is involved in the maintenance of peripheral tolerance. Hence, genetically determined failure of AICD of autoreactive T cells has been suggested to be a cause of MS (26). This hypothesis is supported by reports demonstrating derangements of the Fas system in the pathogenesis of this disorder in terms of increased cerebrospinal fluid and serum levels of soluble Fas protein and a functional deficit in Fas-induced elimination of T cells in MS patients (27–29). Thus, cell death triggered
by Fas appears to constitute an integral part of MS regulation, and emerging evidence indicates that Fas-mediated apoptosis of activated T cells and other inflammatory cells in the CNS is also important for controlling and terminating the inflammatory response in EAE (30–35).

Sensitivity to Fas-mediated apoptosis can be modulated by various antiapoptotic proteins such as the Fas-associated death domain-like IL-1ß-converting enzyme inhibitory proteins (FLIPs). FLIP was initially detected as a gene product of several γ-herpes viruses (36). Subsequently, cellular FLIP (c-FLIP) was identified in both humans and mice (37). Although human FLIP comprises a family of at least 11 different splice forms at the mRNA level (38), it appears to be expressed mainly as long (FLIP_L) and short forms at the protein level (39). FLIP is a potent inhibitor of apoptosis induced by Fas and other death receptors of the TNFR superfamily (40). It is recruited into death receptor signaling complexes and prevents the activation of procaspase-8, disconnecting receptor signaling from the caspase cascade that executes cell death (41).

The importance of c-FLIP as a regulator of T cell apoptosis has been illustrated by detection of high levels of c-FLIP in naive Fas-resistant lymphocytes, followed by a specific down-regulation of this molecule upon AICD (37). Hence, constitutive expression of c-FLIP in lymphocytes by retroviral gene transfer was able to rescue activated B and T cells from apoptosis and resulted in autoimmunity as a consequence of impaired elimination of autoreactive lymphocytes (42). Thus, c-FLIP plays a role in the maintenance of immunological tolerance, and abnormal expression of this protein may contribute to the development and progression of autoimmune diseases by extending the viability of pathogenic self-reactive lymphocytes. This theory is interesting in view of recent data reporting overexpression of the long and short forms of c-FLIP in intrathoracic lymphocytes from patients with MS (43). A specific up-regulation of these two isoforms in activated T cells from patients with clinically active MS, correlating with resistance to Fas-mediated apoptosis, has also been demonstrated (44).

Based on these observations, we sought to assess the effects of overexpression of human FLIP_L (hFLIP_L) in MOG-induced EAE in DBA/1 mice. This was achieved by using a retrovirus-based gene delivery system to constitutively express hFLIP_L in the whole blood lineage of cells. In the present study, we found that overexpression of hFLIP_L in vivo by retroviral transduction of hematopoietic stem cells led to exacerbation of EAE, featured by severe acute and chronic phases with increased mortality when compared with control mice. This appeared to be caused by a heightened and prolonged inflammatory response in the CNS of the FLIP animals.

Materials and Methods

Mice

Female DBA/1 mice (H-2^d^) of 8–10 wk were purchased from The Jackson Laboratory (Bar Harbor, ME) or from Taconic M & B (Ry, Denmark). All mice were kept at the animal facility of the Stockholm University. All experiments performed were in accordance with the relevant ethical committee in North Stockholm.

Production of retroviral particles

The murine stem cell virus (MSCV)-hFLIP_L/ internal ribosome entry site (IRES)-green fluorescent protein (GFP) construct was generated as described (38). Empty (mock) or FLIP-harboring MSCV-IRES-GFP retroviral expression vectors were used to transiently transfect the phoenix-eco packaging cell line using the Lipofectamine 2000 Reagent (Life Technologies, Paisley, U.K.). Supernatants containing recombinant viral particles were harvested after 48 or 72 h and subsequently used for transduction of bone marrow cells.

Bone marrow stem cell enrichment and retroviral transduction

Bone marrow was extracted from the femur, tibia, and humerus of mice 48 h after i.p. treatment with 150 mg/kg of 5-fluorouracil (Fluorouracil, Pharmacia, Stockholm, Sweden). Bone marrow cells were enriched for hemopoietic progenitors and stem cells by negative selection using the StemSep kit (StemCell Technologies, Vancouver, British Columbia, Canada), according to the manufacturer’s specifications. Cells were cultured for 48 h in OPTI-MEM I supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, 3% IL-3 (supernatant from transfected X63 cells) (45), 3% stem cell factor-containing supernatant (kind gift from A. Cumano, Centre National de la Recherche Scientifique Unite de Recherche Associee 1961, Pasteur Institute, Paris, France), 3% of a supernatant from S17 stromal cells (46), and 1% IL-6 (supernatant from T24 bladder carcinoma cells). The stem cells and progenitors were then subjected to spin infections with control (mock) or hFLIP,-containing retroviruses. Briefly, cells were mixed with viral supernatant in the presence of 6 μg/ml Polybrene (Sigma-Aldrich, St. Louis, MO) and centrifuged at 780 × g for 45 min. Further virus supernatant was then added, and cells were centrifuged at 780 × g for an additional 45 min. The procedure was repeated three times at 24-h intervals. After the last spin infection, cells were cultured for another 72 h and analyzed for expression of GFP by flow cytometry using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ). Transplantation was performed by i.v. injection of 1 × 10^6 cells into lethally irradiated (960 rad) syngeneic recipient mice. Irradiated mice were then maintained on 8 μg/ml of doxycyclin (doxycyclin; Merckle, Blaubeuren, Germany) in sterile filter-sterilized cups in isolators for 4–6 wk to prevent opportunistic infections during reconstitution. Later, PBLs from mock and FLIP mice were examined for GFP expression by flow cytometry (described below) to assess donor-specific hemopoietic reconstitution.

Stimulation of T cells and Western blot analysis

Splenocytes from mock and FLIP mice were cultured for 72 h at a density of 2 × 10^6 cells/ml with 2 μg/ml Con A (Amersham Pharmacia Biotech, Uppsala, Sweden) and 10 U/ml of IL-2 (supernatant from transfected X63 cells) (45) in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 10 nM HEPES, 1 mM sodium pyruvate, and 50 μM 2-ME. Cells were then washed and cultured in the presence of 10 U/ml of IL-2 for another 48 h. Viable cells were purified over Lympholyte-M gradient (Cedarlane Laboratories, Hornby, Canada), as recommended by the manufacturer. Cytosolic protein extracts from the Con A-stimulated splenocytes as well as mock and hFLIP,-transduced A20 B lymphoma cells were obtained, as described previously (38). Lysates were then analyzed by 12.5% SDS-PAGE and blotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech) using standard procedures. Detection of human FLIP was conducted as described using rat anti-human FLIP (Dove-2) mAb (Alexis Biochemicals, Lausen, Switzerland) (38). Quantification of the expression of FLIP_L was performed using the ImageQuant software version 5.0.

Apoptosis assay

AICD of T cells was induced by culturing splenocytes from mock and FLIP mice with Con A and IL-2, as described above. Viable cells were purified over Lympholyte-M gradient, and Fas-mediated apoptosis was triggered on day 5 by stimulation of the activated T cells at 0.5 × 10^6 cells/ml with 2-fold dilutions of soluble Fasl supernatant from transfected phoenix-ampho cells and 10 U/ml of IL-2 or IL-2 alone in supernatant from nontransfected phoenix-ampho cells for 24 h (38). Apoptosis was detected by staining the cells with propidium iodide and annexin V-biotin, followed by streptavidin-allophycocyanin, according to the manufacturer’s instructions (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany). The samples were then analyzed on a FACS Calibur flow cytometer (BD Biosciences) using CellQuest software.

Induction and clinical evaluation of EAE

Mice were anesthetized and injected intradermally at the base of the tail with 200 μl inoculum composed of 50 μg of rMOG_E125 in sodium acetate buffer (pH 3.0) emulsified (1:1) with CFA containing 300 μg of Mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, MI). Mice were scored for clinical signs of EAE and weighed daily. The score was designated as follows: 0, no disease signs; 1, limp tail; 2, waddling; 3, hind limb weakness and abnormal gait; 4, partial hind limb paralysis; 5, complete hind limb paralysis; 6, forelimb paralysis or weakness; 7, moribund or dead.
Extraction of cells from the CNS

Spinal cords and brains were collected from mock and FLIP mice, and cells were isolated from the homogenized CNS tissues using Percoll (Amersham Pharmacia Biotech) density-gradient centrifugation. Briefly, the brain and spinal cord tissues were homogenized in 5 ml of 50% Percoll gradient (HBSS, 0.1% BSA, 1% glucose, and 1.070 g/ml Percoll) supplemented with 100 U/ml of DNase type I (Boehringer Mannheim, Scandanavia AB, Bromma, Sweden). The homogenates were then underlaid with 3.5 ml of 65% Percoll gradient (density 1.087 g/ml) and subsequently overlaid with 5 ml of 30% Percoll gradient (density 1.045 g/ml), followed by centrifugation at 1000 x g for 30 min at 4°C. Cells were extracted from the 50/63% (lymphoid) and 30/50% (myeloid) Percoll interphases, respectively, and were then washed once in PBS containing 0.1% BSA and 1% glucose and centrifuged at 600 x g for 15 min at 4°C before subsequent analyses.

Isolation of PBLs and flow cytometry analysis

Mice were bled, and PBLs were isolated from heparinized blood diluted in PBS by density-gradient centrifugation on Lymphoprep (Nycomed Pharma AS, Oslo, Norway). PBLs or cells isolated from the CNS were washed in PBS containing 2% FCS and 0.01% azide before staining for flow cytometry. The following Abs from BD PharMingen (San Diego, CA) or Caltag Laboratories (Burlingame, CA) were used: PE-labeled anti-CD4, anti-CD8, anti-CD3, anti-CD22, and anti-CD11b (Mac-1α chain); allophycocyanin-labeled anti-CD44 and anti-CD45; biotinylated anti-Ly-6G (GR-1); and allophycocyanin-conjugated streptavidin. Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences), and viable nucleated cells were determined by gating in forward and side scatter and by exclusion of propidium iodide-positive cells. For each sample, at least 5000 events within this population were acquired, and the results were analyzed using CellQuest software.

ELISA

Splenocytes from mock and FLIP mice were stimulated at 2.5 x 10^6 cells/ml with 8 μg/ml of plate-bound anti-CD3ε Ab or 50 μg/ml of MOG35-152. Cell culture supernatants were collected 48 h later, and concentrations of IFN-γ and IL-4 were assessed by ELISA (Mabtech AB, Nacka, Sweden). The cytokine concentrations in the cell culture supernatants were calculated by extrapolation from the linear portion of the standard curves.

Real-time quantitative PCR analysis

Total RNA from splenocytes or cells extracted from the CNS was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA). cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) in a final volume of 50 μl, according to the manufacturer’s protocol, with random hexamers as primers. The reaction was conducted at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. The various primer and probe combinations allowing amplification of IFN-γ, TNF-α, IL-4, and 18S rRNA cDNAs were purchased as Pre-Developed TaqMan Assay Reagents (Applied Biosystems). The 18S rRNA served as an endogenous control to standardize the amount of sample cDNA added to a reaction. The TaqMan Universal PCR Master Mix (Applied Biosystems) was used for the reaction mixtures of 25 μl, as recommended by the manufacturer. Real-time PCR was performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems) using the Sequence Detection Systems software version 1.7. The conditions for the PCR amplification were: 1 cycle at 50°C for 2 min and 95°C for 10 min, followed by 50 cycles (for IFN-γ, TNF-α, and IL-4) or 40 cycles (for 18S rRNA) of 95°C for 15 s and 60°C for 1 min. Relative amounts of the three different cytokines were calculated using the comparative Cq method.

Results

Generation of hFLIP_L-expressing DBA/1 mice by retroviral transduction of bone marrow stem cells

To be able to address the role of FLIP in EAE disease progression, lethally irradiated DBA/1 mice were reconstituted with syngeneic bone marrow stem cells and precursors overexpressing hFLIP_L or GFP (mock) by means of retroviral gene transfer using the MSCV-IRES-GFP vector system. The MSCV-IRES-GFP retroviral expression vector produces a bicistronic mRNA encoding the gene of interest coupled to enhanced GFP, which serves as a marker of transduction and allows for the monitoring of cells carrying the gene. Approximately 7 wk after transplantation, mice were bled, and the distribution as well as GFP expression of lymphocytes in PBLs were examined by flow cytometry to determine donor-derived hemopoietic reconstitution. As apparent in Fig. 1, the lymphocyte compositions and GFP expressions in the blood of mock and FLIP mice were comparable. The PBLs consisted primarily of CD4+ T cells and B220+ B cells, accounting for 30 and 40% of the cells, respectively (Fig. 1A). The percentage of CD8+ T cells was lower and attained 4% (Fig. 1A). These values were similar to those of wild-type (wt) DBA/1 mice (data not included) and were thus considered to be within normal range. Although the proportion of GFP+ cells among B and T cells was moderately higher in mock animals (60–90%) compared with FLIP animals (40–66%) (Fig. 1B), many lymphocytes sustained expression of GFP in both mouse types, indicating that hemopoiesis was driven to a considerable extent by retrovirally transduced bone marrow stem cells and progenitors. GFP+ lymphoid and myeloid cells were also present in 1-year-old mock and FLIP DBA/1 mice (data not included), demonstrating successful retroviral transduction of stem cells with both short-term and long-term reconstitutive potential.

Retrovirus-mediated expression of hFLIP_L in activated lymphocytes of DBA/1 mice inhibits Fas-induced apoptosis

Retroviral expression of hFLIP_L in FLIP DBA/1 mice was assessed by Western blot analysis of AICD-primed T cells using a mAb to human FLIP (Dive-2). Splenocytes from mock and FLIP animals were sensitized to Fas-mediated apoptosis by stimulation with Con A and IL-2 for 3 days, followed by IL-2 for 2 days to trigger up-regulation of the FasR and a simultaneous down-regulation of endogenous mouse FLIP_L. As anticipated, activated T cells from FLIP mice exhibited a strong expression of FLIP_L due to retrovirus-mediated constitutive expression of this protein (Fig. 2A). Comparison of band intensities revealed a 3.5-fold higher expression of FLIP_L in these T cells compared with T cells from wt and mock animals. Furthermore, retrovirally expressed hFLIP_L...
interfered with AICD of these T cells, as apoptosis-resistant GFP⁺ T cells from FLIP mice accumulated among live cells following exposure to soluble FasL in contrast to GFP⁺ T cells from mock mice (Fig. 2B). The weak bands observed in activated T cells of wt and mock DBA/1 mice in Fig. 2A most likely represent residual levels of endogenous FLIPL, as Dave-2 is known to cross-react with mouse FLIPL (38). Detectable protein levels of murine FLIPL in these animals may reflect insufficient T cell activation because previous findings show expression of FLIP during early stages of T cell activation, but not during late stages (37).

Overexpression of hFLIPL in DBA/1 mice exacerbates MOG-induced EAE

To induce EAE, mock and FLIP DBA/1 mice were immunized with 50 μg MOG in CFA. The disease incidence was 100%, and mice normally developed signs of EAE 11 days postimmunization (Table I). As depicted in Fig. 3, the disease course was acute and chronic nonrelapsing, and the animals reached the peak of the disease ~5 days after onset of EAE, as in wt DBA/1 mice (8). Although both groups exhibited similar score profiles at early stages of the disorder, FLIP mice soon developed an exacerbated form of EAE characterized by a severe acute and chronic phase generally without clinical remission (Fig. 3A). Consequently, the mean maximal disease score was higher for FLIP than mock mice (6.0 vs 5.2), as was the cumulative score (140 vs 107) (Table I). The difference in disease severity between mock and FLIP animals remained evident for as long as 57 days after priming, and was even slightly increased with time (Fig. 3B). Furthermore, the survival rate among FLIP mice was strongly reduced compared with mock mice as a consequence of the more aggressive course of EAE in the FLIP group (Fig. 3C). Hence, as many as 12 of 28 (42.8%) FLIP mice in contrast to 4 of 30 (13.3%) mock mice died from EAE, particularly during the acute phase of the disease (Table I). Despite prominent differences in EAE progression between mock and FLIP animals, comparable amounts of MOG-specific IgG were detected in the sera of these animals when examining autoantibody responses to MOG₁–₁₂₅ by ELISA (data not included).

Constitutive expression of hFLIPL in DBA/1 mice leads to an enhanced and prolonged inflammatory response in the CNS during EAE

To gain some insight into the cellular events associated with the exacerbation of EAE in FLIP DBA/1 mice, cells were isolated from brain and spinal cord homogenates of mock and FLIP mice by Percoll gradient centrifugation 16 or 21 days after MOG immunization. Inflammatory cells were obtained from two different Percoll interphases that were analyzed separately for the presence of lymphoid (L-phase) or myeloid (M-phase) markers by flow cytometry.

An increased cellular infiltration in the CNS of FLIP animals relative to mock animals was observed, particularly at day 16, when estimating the number of viable white blood cells in the CNS samples by vital staining or when comparing the proportion of live nucleated cells between mock and FLIP samples during flow cytometry analysis (data not included). The cellular compositions of CNS infiltrates from mock and FLIP mice are presented in Fig. 4. The number of CD4⁺, B220⁺, and Mac-1⁺ cells in Fig. 4. A–C, was acquired by calculating the number of GFP⁺ (filled portions of the bars) and GFP⁻ cells in these populations per sample of 10⁵ live nucleated cells. The pattern of CD4⁻ T cell expansion in the CNS of mock and FLIP mice during EAE indicated a higher proportion of these cells in the acute phase (day 16) than in the chronic phase (day 21) of the disease (Fig. 4A). The decline in CD4⁺ T cells at day 21 was much less evident in FLIP mice compared with that in mock mice. Similarly, the decrease of GFP⁺ T cells from days 16 to 21 was considerably lower in FLIP than mock animals (Fig. 4A). Thus, a significant number of CD4⁺ T cells expressing hFLIPL appeared to remain in the CNS of FLIP mice during the chronic phase of the disease, indicating a defective clearance of these cells. When studying the frequency of activated CD4₁⁺CD44highCD4⁻ T cells in the CNS of mock and FLIP mice at day 16, the latter displayed a 2-fold increase of such cells compared with mock mice among all CD4⁺ T cells (24.3 vs 11.1%) and

**Table I. Susceptibility of mock and FLIP DBA/1 mice to MOG-induced EAE**

<table>
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<th>DBA/1 Mice</th>
<th>Incidence</th>
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<th>Mean Maximum Severity</th>
<th>Mortality</th>
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<td>11.5</td>
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<td>12/28</td>
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**FIGURE 2.** Activated splenocytes from FLIP mice overexpress hFLIPL and are refractory to Fas-mediated apoptosis. T cells were primed for AICD by stimulating splenocytes from wt, mock, or FLIP DBA/1 mice with 2 μg/ml Con A and 10 U/ml IL-2 for 72 h, followed by 10 U/ml IL-2 for 48 h. A, Expression of FLIPL in activated splenocytes of these mice was analyzed by Western blot using the Dave-2 mAb against human FLIP. Mock and hFLIPL-transduced A20 B lymphoma cells served as negative and positive controls, respectively. Quantification of the expression of FLIPL was performed using the ImageQuant 5.0 software. Comparison of retrovirally expressed hFLIPL in FLIP mice with endogenous levels of mouse FLIPL in wt and mock mice is only valid assuming that the affinity of Dave-2 for human and mouse FLIPL is similar. B, AICD was induced by treating activated splenocytes from mock and FLIP mice with 2-fold dilutions of soluble FasL supernatant from phoenix-ampho cells and 10 U/ml IL-2 or IL-2 alone for 24 h. Apoptosis was detected by double staining with annexin V and propidium iodide before flow cytometry analysis. Fas-mediated apoptosis was successfully induced in both mock and FLIP samples, but the percentage of apoptotic GFP⁺ cells could not be assessed because GFP expression was lost in dying cells. Instead, the percentage of GFP⁻ cells among viable cells was determined by gating on annexin V⁻ and propidium iodide⁻ cells. The percentages of GFP⁺ cells among live cells for IL-2-treated mock and FLIP control samples were 33 and 36%, respectively. Data presented are representative of two experiments.
among GFP<sup>+</sup>CD4<sup>+</sup> T cells (20.8 vs 10.0%) (Table II). This difference was even more pronounced among GFP<sup>+</sup>CD4<sup>-</sup> T cells, with activated T cells constituting 33% of this population in FLIP mice, but only 12.6% in mock mice (Table II). Infiltrating B220<sup>+</sup> B cells were also detected in the CNS of mock and FLIP animals 16 and 21 days after MOG immunization (Fig. 4B). As was the case for the T cells, there was an impaired reduction of B cells in FLIP mice at day 21. In fact, the B cells rather increased with time, accompanied by a marked expansion of GFP<sup>+</sup> B cells rather than increased with time, accompanied by a marked expansion of GFP<sup>+</sup> B cells within this population from days 16 to 21 (Fig. 4C). Although not included in Fig. 4, small numbers of CD8<sup>+</sup> T cells were found in the CNS samples of mock and FLIP mice, but the levels were too low to assess any significant differences between the two groups.

Macrophages, granulocytes, and microglia share the same surface marker (Mac-1). Mac-1<sup>+</sup> macrophages and granulocytes were therefore distinguished by staining with GR-1, and blood-derived macrophages were discriminated from CNS-resident microglia by staining Mac-1<sup>+</sup> cells with CD45. Granulocytes (primarily neutrophils) were thus Mac-1<sup>+</sup>GR-1<sup>-</sup>, macrophages were Mac-1<sup>+</sup>CD45<sup>high</sup>, and microglia were Mac-1<sup>+</sup>CD45<sup>low</sup>, according to previously published results (47, 48). The number of cells for each of these populations was calculated from the percentages of these cells among Mac-1<sup>+</sup>GFP<sup>+</sup> and Mac-1<sup>+</sup>GFP<sup>-</sup> cells (Fig. 4, D-F). Analysis of Mac-1<sup>+</sup> cells revealed a drastic increase of such cells in the CNS of FLIP animals at day 16 compared with mock mice (Fig. 4C). Subdivision of Mac-1<sup>+</sup> cells into granulocytes, macrophages, and microglia demonstrated an 8-fold increase of granulocytes and a 6-fold increase of macrophages in the CNS of FLIP mice relative to mock mice, but no major difference in the amount of microglia, except for a somewhat higher proportion of GFP<sup>-</sup> microglia in the FLIP animals (Fig. 4, D-F). Despite the remarkable infiltration of granulocytes and macrophages in the FLIP mice, no pronounced enrichment for GFP<sup>+</sup> cells in these populations was observed at day 16 (Fig. 4, D and E), implying that the extensive proliferation of Mac-1<sup>+</sup>GFP<sup>+</sup> cells during the acute phase of EAE may be indirectly affected by other cells overexpressing hFLIP<sub>L</sub>.

**DBA/1 mice overexpressing hFLIP<sub>L</sub> exhibit increased levels of TNF-α in the CNS during the acute phase of EAE**

There is a possibility that the increased inflammatory response in FLIP animals is paralleled by an elevated production of proinflammatory cytokines. To test this concept, the Th1 (IFN-γ and TNF-α) and Th2 (IL-4) cytokine profiles in the CNS of mock and FLIP DBA/1 mice were studied by real-time quantitative PCR. Relative mRNA levels for IFN-γ, TNF-α, and IL-4 in the CNS L- and M-phases at days 16 and 21, respectively, were calculated using a wt DBA/1 spleen calibrator according to the comparative C<sub>T</sub> method (Table III). The kinetics of IFN-γ, TNF-α, and IL-4 production in mock and FLIP mice peaked at day 16, and then decreased by day 21 (Table III). Interestingly, the relative amount of TNF-α mRNA in the M-phase at day 16 was ~3-fold higher in FLIP (2.59) than in mock mice (0.92). This may be the result of the substantial infiltration of macrophages in the CNS of FLIP animals during the acute phase of EAE (Fig. 4E). In contrast to TNF-α, no significant differences in IFN-γ or IL-4 expression were recorded between mock and FLIP mice, although the relative amount of IFN-γ mRNA was enhanced in the L-phase of mock animals compared with FLIP animals at day 16 (Table III). However, this difference (0.34 vs 0.15) may not be reliable given the wide range...
(0.15–0.76) of the relative amounts of IFN-\(\gamma\) transcripts in mock mice.

**FLIP DBA/1 mice display Th1 skewed peripheral cytokine responses during the chronic phase of EAE**

The cytokine patterns in the spleens of mock and FLIP mice were analyzed 16 and 21 days after challenge with MOG to assess whether the difference in the inflammatory response between these animals was discernible beyond CNS tissues. The amount of IFN-\(\gamma\) and IL-4 produced by anti-CD3- or MOG-stimulated splenocytes of individual mice was quantified by ELISA, and the IFN-\(\gamma\)/IL-4 ratio was established. Determination of the Th1/Th2 cytokine ratio could only be performed for the spleen cultures primed with anti-CD3 Ab, as IL-4 was below detection limits in the MOG-stimulated cultures. As depicted in Fig. 5A, FLIP mice displayed a rather strong Th2 phenotype at day 16 caused by slightly reduced levels of IFN-\(\gamma\) combined with elevated levels of IL-4 in these mice compared with mock mice. The Th2 cytokine bias among FLIP animals during the acute phase of the disease was unexpected, but consistent with the real-time PCR findings showing a high production of IL-4 in the CNS of these animals at day 16 (Table III). At day 21, FLIP mice revealed a significant Th1 cytokine bias when compared with mock mice (Fig. 5A). The overall higher IFN-\(\gamma\)/IL-4 cytokine ratios in the FLIP group were due to an increased production of IFN-\(\gamma\) and a concomitant decrease in IL-4 (data not included). Examination of MOG-specific cytokine responses also demonstrated an increased production of IFN-\(\gamma\) in FLIP mice relative to mock mice at that time point (Fig. 5B). The elevated levels of IFN-\(\gamma\) observed in both anti-CD3- and MOG-stimulated spleen cultures of FLIP animals at day 21 may result from a defective down-regulation of the inflammatory response during late stages of EAE, causing the severe chronic phase without clinical remission associated with these mice.

**Discussion**

In this study, we have demonstrated that constitutive expression of hFLIP\(_{\text{L}}\) in DBA/1 mice by retroviral gene transfer of hemopoietic stem cells leads to exacerbation of MOG-induced EAE. The exacerbation was evident by severe acute and chronic phases, resulting in a high mortality among these animals. The aggravated course of EAE in FLIP mice was associated with an enhanced inflammatory response in the CNS at the height of disease, characterized by a prominent expansion of macrophages and granulocytes and an elevated production of TNF-\(\alpha\). Furthermore, a defective clearance of CD4\(^+\) T cells was observed in FLIP mice compared with mock mice. The increased inflammatory response in the CNS of FLIP mice was accompanied by a higher number of activated CD4\(^+\) T cells, as determined by the percentage of CD44 high CD4\(^+\) T cells among GFP\(^+\) CD4\(^+\) T cells (Table II). The increased production of IFN-\(\gamma\) and TNF-\(\alpha\) in these animals was consistent with the clinical and pathological findings, indicating a severe inflammatory response associated with the severe chronic phase of EAE.
The mRNA levels for the different cytokines were normalized to 18S rRNA levels and related to a wt DBA/1 spleen calibrator according to the comparative C_T method. The values within parentheses indicate the range for a given relative amount of cytokine mRNA based on the SD of triplicate samples.

<table>
<thead>
<tr>
<th>DBA/1 Mice</th>
<th>Days Postimmunization</th>
<th>Tissue</th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>IL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock 16</td>
<td>CNS L-phase</td>
<td></td>
<td>0.34</td>
<td>(0.15–0.76)</td>
<td>3.32</td>
</tr>
<tr>
<td>FLIP 16</td>
<td>CNS L-phase</td>
<td></td>
<td>0.15</td>
<td>(0.12–0.19)</td>
<td>3.10</td>
</tr>
<tr>
<td>Mock 21</td>
<td>CNS L-phase</td>
<td></td>
<td>0.17</td>
<td>(0.14–0.22)</td>
<td>0.81</td>
</tr>
<tr>
<td>FLIP 21</td>
<td>CNS L-phase</td>
<td></td>
<td>0.18</td>
<td>(0.06–0.53)</td>
<td>0.50</td>
</tr>
<tr>
<td>Mock 16</td>
<td>CNS M-phase</td>
<td></td>
<td>0.32</td>
<td>(0.26–0.39)</td>
<td>0.92</td>
</tr>
<tr>
<td>FLIP 16</td>
<td>CNS M-phase</td>
<td></td>
<td>0.28</td>
<td>(0.20–0.38)</td>
<td>2.59</td>
</tr>
<tr>
<td>Mock 21</td>
<td>CNS M-phase</td>
<td></td>
<td>0.02</td>
<td>(0.01–0.04)</td>
<td>0.14</td>
</tr>
<tr>
<td>FLIP 21</td>
<td>CNS M-phase</td>
<td></td>
<td>0.06</td>
<td>(0.04–0.08)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

a The mRNA levels for the different cytokines were normalized to 18S rRNA levels and related to a wt DBA/1 spleen calibrator according to the comparative C_T method. The values within parentheses indicate the range for a given relative amount of cytokine mRNA based on the SD of triplicate samples.

b Not detected, C_T > 50.

and B cells from the CNS of these animals was observed during the chronic stage of the disorder. The extended inflammatory reaction in the CNS was also visible in the periphery as a Th1 cytokine bias in the spleen, caused by an increased production of IFN-γ and a concomitant decrease of IL-4.

Despite previous reports suggesting that cellular overexpression of FLIP is a feature of clinically definite MS (43, 44), the effect of FLIP overexpression in EAE models has never been evaluated before. We therefore tested the consequence of constitutive FLIP expression in the pathogenesis of MOG-induced EAE in DBA/1 mice. Our study demonstrates that expression of a potent inhibitor of death receptor-mediated apoptosis in inflammatory cells aggravates the progression of EAE in mice. This finding strengthens the hypothesis that death receptor-induced apoptosis constitutes a significant part of the regulation and subsidence of inflammation in EAE. A role for FasL in recovery from EAE has been demonstrated previously by performing adoptive transfers of myelin-primed nontransgenic or myelin basic protein (MBP)-specific TCR transgenic wt, lpr, or gld lymphocytes into congenic wt, lpr, and gld recipient mice (49). It was deduced from these experiments that FasL, expressed by the host is very important for clinical remission, as many gld mice receiving wt lymphocytes exhibited an extended disease course. A similar conclusion was drawn when transfer of cells from an MBP-specific TCR transgenic T cell line to gld recipient mice caused more severe disease course than in wt recipients (50). Further evidence for a role of FasL in the recovery of EAE was provided by experiments using a DNA vaccine encoding this protein (51). Naked DNA vaccination generated protective immunity against this product in the form of FasL-specific autoantibodies. Administration of these autoantibodies to MBP-treated rats after the peak of disease delayed recovery. This was partially caused by protection of T cells and macrophages from apoptosis, which resulted in the persistence of these cells at the site of inflammation.

Although Fas-mediated apoptosis seems to play a major role in the clearance of activated immune cells in EAE, some reports claim that deletion of T cells is foremost Fas and FasL independent because it occurs in lpr and gld mice (52, 53). Clearly, additional apoptosis mechanisms are operative in this disease. Factors such as glucocorticoids (54) and NO (55) trigger apoptosis of infiltrating T cells in EAE. Furthermore, a new death receptor-induced cell death pathway that is caspase independent, but relies on the Fas-associated death domain adaptor protein and the kinase receptor-interacting protein, has been described and appears to efficiently contribute to the killing of activated T cells (56). Passive cell death caused by cytokine deprivation is another form of apoptosis that occurs when activated T cells do not receive sufficient costimulation to maintain adequate levels of IL-2. This type of cell death exerts its effect through the mitochondria and is regulated by members of the Bcl-2 family (57). The importance of passive cell death in the pathogenesis of EAE was recently shown in mice transgenic for the antiapoptotic molecule Bcl-xL in the T cell repertoire (58). These animals developed a severe EAE with an increased activation of T cells, a more grave pathology, and a reduced rate of apoptosis in the CNS compared with wt littermates. This work not only underscores the magnitude of T cell apoptosis in terminating the inflammatory response in EAE, but also indicates the necessity for proper regulation of Bcl-2-related proteins, as exemplified in

**Figure 5.** Peripheral cytokine responses are Th1 polarized in FLIP mice during the chronic phase of EAE. Spleen cells from MOG-injected mock and FLIP mice were stimulated with 8 μg/ml anti-CD3ε Ab or 50 μg/ml MOG for 48 h. Production of IFN-γ and IL-4 in the supernatants of these cultures was assessed and quantified by ELISA. A, The IFN-γ/IL-4 balance in anti-CD3-primed spleen cultures of mock and FLIP animals was examined 16 and 21 days postimmunization. C, designate individual mice. The horizontal bars represent the means of respective groups. The differences between the two groups did not reach statistical significance. Data in A, but not B, are shown for two pooled experiments.
another study showing abnormal expression patterns of such proteins in lymphocytes from MS patients (59).

The fact that numerous apoptosis pathways are used in EAE to limit inflammation may explain the lack of a drastic expansion of GFP\(^+\) cells in the CNS of FLIP mice during the acute and chronic phases of the disease. Because hFLIP\(_L\) selectively prevents death receptor-mediated apoptosis, it could not confer complete protection from deletion of T cells in the CNS. Hence, CD4\(^+\) T cells were eliminated in FLIP mice, but to a lesser extent than in mock mice during the chronic stage of EAE. Nevertheless, our results clearly indicate an advantage for survival of GFP\(^+\) cells expressing hFLIP\(_L\) compared with GFP\(^+\) cells, because the decline in GFP\(^+\) T cells was much lower than that of GFP\(^-\) T cells in the CNS of diseased FLIP animals. The slight reduction of GFP\(^+\) T cells in these mice was perhaps in part due to a recirculation of cells to peripheral lymphoid organs, as this has been suggested to be a mechanism for clearance of nonautoimmune T cells from the CNS during spontaneous disease remission (22). Interestingly, an impaired decrease of GFP\(^-\) T cells was also observed in the CNS of FLIP mice 21 days after MOG immunization, which may result from an indirect positive effect of hFLIP\(_L\)-expressing cells on these T cells.

Despite similar numbers of CD4\(^+\) T cells in the CNS of mock and FLIP mice at the peak of the disease, the proportion of activated CD4\(^+\)CD44\(^hi\)/CD44\(^-\) T cells was higher in the FLIP animals. Furthermore, these activated and potentially encephalitogenic T cells were enriched within the GFP\(^+\) T cell pool, albeit to a lesser degree than anticipated. Earlier studies performed in rats have reported that apoptosis during recovery from EAE is predominantly confined to myelin-reactive T cells and that bystander-activated T cells recirculate to the periphery (21, 22, 60). Assuming that apoptosis of such cells is largely Fas mediated, any accumulation of activated myelin-specific GFP\(^+\) T cells in the FLIP mice would have been rather discrete, as only very few T cells infiltrating the CNS are believed to be autoreactive (61, 62). The conclusion that apoptosis solely affects myelin-reactive T cells has later been challenged by the finding that T cells die by apoptosis regardless of their Ag specificities or activation states (63).

A significant expansion of B cells was detected in the CNS of MOG-treated FLIP mice during EAE, and these cells did not decrease after 21 days as in mock mice. A role for B cells in the pathogenesis of EAE has recently been demonstrated in DBA/1 mice immunized with MOG (6, 8). These animals experienced an extensive demyelination in the CNS during EAE progression that was shown to be dependent on the presence of B cells and the production of autoreactive Abs. The fate of B cells in the spontaneous recovery from EAE is rather unclear, but new findings propose that they are eliminated through self-inflicted Fas-FasL interactions at the site of inflammation (35). Thus, the expansion of GFP\(^+\) B cells in the CNS of FLIP mice during late stages of EAE probably reflects the apoptosis-counteracting effect of hFLIP\(_L\), and may have promoted further autoantibody-driven demyelination that in turn could have contributed to the severe clinical signs characterizing the chronic disease phase of these animals. Even though autoantibody production may have been higher in the CNS of FLIP mice compared with mock mice during the course of EAE, serum levels of anti-MOG IgG did not differ between these animals during the acute or the chronic phase of the disease.

Another component of the inflammatory response in MOG-immunized FLIP mice was the remarkable increase of Mac-1\(^+\) cells in the CNS during the acute phase of EAE. The increase essentially comprised macrophages and granulocytes/neutrophils and, in contrast to the delayed clearance of T and B cells, the number of these cells had decreased considerably as the disease entered the chronic phase. The low proportion of GFP\(^+\) cells among Mac-1\(^+\) cells and the efficient deletion of both GFP\(^+\) and GFP\(^-\) cells during the chronic stage of EAE suggest that these inflammatory cells did not benefit directly from the protective effect of hFLIP\(_L\), as they were most likely killed by factors other than death receptors (64). The great abundance of macrophages and granulocytes in the CNS of FLIP mice at the peak of disease was therefore probably indirectly influenced by other cells expressing hFLIP\(_L\), but may have been of prime importance for the exacerbation of EAE in these animals.

The latter conclusion concords with other reports demonstrating a critical role for macrophages and granulocytes in the pathogenesis of EAE, in which the degree of infiltration of such cells in the CNS was closely related to the clinical severity of the disorder (65–68). We have also reported a critical role of Fc\(_\gamma\)R, which are expressed by macrophages and microglia, in MOG-induced EAE (7). Although the fraction of GFP\(^+\) cells within the microglia population was very low, it was much higher in FLIP than mock mice. This is interesting considering the finding that TGF-\(\beta\) can promote up-regulation of FLIP, and thereby protect microglia from sensitization to FasL-mediated apoptosis by IFN-\(\gamma\) and TNF-\(\alpha\) (69). Because FLIP appears to be important for the survival of microglia during inflammation, it may explain the selective expansion of GFP\(^+\) microglia in the FLIP mice at the height of disease.

The network of cytokines expressed in EAE is highly complex. Whereas proinflammatory Th1 cytokines drive the disease, Th2 cytokines are believed to diminish the inflammatory response in EAE (70). The elevated production of TNF-\(\alpha\) in the CNS of FLIP mice during the acute phase of EAE was perhaps a consequence of the extensive expansion of macrophages at that time point and may have been an important constituent in disease exacerbation. Furthermore, the Th1 cytokine deviation in the spleens of these animals indicated a defective down-regulation of the inflammatory response that may also have contributed to aggravation of the disease. The similar expression of IFN-\(\gamma\) and IL-4 in the CNS of mock and FLIP mice was surprising given the observed differences in the periphery, but suggests that the expression profile of cytokines is not always comparable between the CNS and peripheral lymphoid organs.

The high levels of IL-4 in the CNS of mock and FLIP animals and the Th2 cytokine bias in the spleens of FLIP mice at the peak of disease are difficult to reconcile with the concept that Th2 cytokines are disease antagonists. However, conflicting results concerning the role of these cytokines in the regulation of EAE have been obtained from several reports. For instance, it was shown that IL-4-deficient mice were able to remit from EAE (71). Another study could not detect any increase of Th2 cytokines in the CNS or in the periphery of animals with relapsing EAE during the recovery phase (72). Instead, up-regulation of such cytokines in the CNS of mice was shown to rather be a feature of the nonrelapsing type of EAE (73). This finding is highly relevant for the elevated levels of IL-4 recorded in our EAE model being chronic nonrelapsing.

Altogether, our results show that inhibition of death receptor-induced apoptosis by retrovirus-mediated expression of hFLIP\(_L\) in immune cells leads to exacerbation of EAE in mice. A consequence using our approach was that overexpression of hFLIP\(_L\) in the immune system probably also encompassed cells with immunoregulatory properties such as CD25\(^+\) CD4\(^+\) T cells (15), CD8\(^+\) T cells (16), dendritic cells (18), NK cells (19), and NK T cells (17). Despite this widespread expression of hFLIP\(_L\), the disease course was still aggravated. This clearly demonstrates the importance for efficient apoptotic deletion of pathogenic inflammatory cells to allow clinical recovery in EAE, and targeted apoptosis of such cells may have future therapeutic value for MS.
References


