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X-linked Inhibitor of Apoptosis Regulates T Cell Effector Function

Simone P. Zehntner,* Lyne Bourbonnière,* Craig S. Moore,§ Stephen J. Morris,† Danielle Methot,‡ Martine St. Jean,† Eric Lacasse,† Andrea L. O. Hebb,§ George S. Robertson,¶ Jon Durkin,† John W. Gillard,‡ and Trevor Owens*§

To understand how the balance between pro- and anti-apoptotic signals influences effector function in the immune system, we studied the X-linked inhibitor of apoptosis (XIAP), an endogenous regulator of cellular apoptosis. Real-time PCR showed increased XIAP expression in blood of mice with experimental autoimmune encephalomyelitis, correlating with disease severity. Daily administration (10 mg/kg/day i.p.) of a 19-mer antisense oligonucleotide specific for XIAP (ASO-XIAP) abolished disease-associated XIAP mRNA and protein expression, and given from day of onset, alleviated experimental autoimmune encephalomyelitis and prevented relapses. Prophylactic treatment also reduced XIAP expression and prevented disease. Random or 5-base mismatched ASO was not inhibitory, and ASO-XIAP did not affect T cell priming. In ASO-XIAP-treated animals, infiltrating cells and inflammatory foci were dramatically reduced within the CNS. Flow cytometry showed an 88–93% reduction in T cells. The proportion of TUNEL-positive apoptotic CD4+ T cells in the CNS was increased from <1.6 to 26% in ASO-XIAP-treated mice, and the proportion of Annexin V-positive CD4+ T cells in the CNS increased. Neurons and oligodendrocytes were not affected; neither did apoptosis increase in liver, where XIAP knockdown also occurred. ASO-XIAP increased susceptibility of T cells to activation-induced apoptosis in vitro. Our results identify XIAP as a critical controller of apoptotic susceptibility of effector T cell function. The Journal of Immunology, 2007, 179: 7553–7560.

Apoptosis of cells is initiated via extrinsic signaling through death receptor complexes and/or via intrinsic mitochondrial release of proapoptotic factors. Inhibitor of Apoptosis (IAP)3 proteins such as X-linked inhibitor of apoptosis (XIAP), cIAP1, and cIAP2 are critical controlling elements that inhibit the apoptotic cascade (1). IAPs directly inhibit the initiators and executioners of cell death, the caspase enzymes, via interactions mediated by or associated with baculovirus inhibitory repeats domains (BIR), an inherent characteristic of IAP structure (reviewed in Ref. 2, 3). XIAP, the most potent and best-characterized IAP family member, inhibits both the intrinsic and extrinsic apoptotic cascades. XIAP is expressed at varying levels in a wide variety of cells. The susceptibility of a cell to an apoptotic signal is dictated in part by the level of XIAP at the time of stimulus. Increased XIAP expression effectively protects cells from death driven by apoptotic signals (4). Conversely, deletion or reduction in the level of XIAP, or interfering with its BIR domains, removes this protection, effectively permitting the cell to execute programmed cell death (5–9).

Regulation of T cell apoptosis plays a critical role in controlling immune responses. Levels of T cell apoptosis influence the immune repertoire and contribute to the maintenance of homeostasis. This has implication for autoimmune disease. There is evidence of aberrant T cell apoptosis in multiple sclerosis (MS) (10–14). This suggests a potential pathogenic mechanism, resulting from the defective apoptotic termination of autoreactive T lymphocytes (15), supported by a number of studies (15–20).

Predisposing autoimmune T cells to apoptose might therefore alleviate disease. Antisense oligonucleotides (ASO), designed to bind XIAP mRNA, direct the RNA transcript for degradation rather than translation (21), and effectively reduce the pool of translated XIAP protein in a variety of cancer cellular models (5, 6, 22–25). We find that substantial elevation of XIAP occurs in myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE), which correlated with disease severity, and that reduction of the elevated levels of XIAP by the specific murine antisense oligonucleotide, AEG35169 (XIAP-ASO), prevents or cures MOG-induced EAE. This mechanism involves increased apoptotic sensitivity of effector T cells and we show that those activated T cells...
infiltrating the CNS undergo selective apoptosis at the site of myelin presentation.

Materials and Methods

Mice

Female C57BL/6 mice were purchased from The Jackson Laboratories. Mice were housed in microisolator cages in a pathogen-free facility. Animal breeding and experimentation was conducted in accordance with guidelines of the Canadian Council on Animal Care, as approved by the McGill University Animal Care Committee.

Induction and monitoring of EAE

EAE was induced by s.c. injection of 100 μg MOG peptide 35–55 (Sheldon Biotechnology Centre), emulsified in CFA (Fisher Scientific), containing 500 μg heat-killed Mycobacterium tuberculosis (H37RA) (Difco). The injection was repeated 7 days later. Mice received 200 ng pertussis toxin (Sigma-Aldrich) on days 0 and 2 by i.p. injection.

Animals were monitored daily for assessment of weight and clinical symptoms. Symptoms of EAE were scored as follows: I: weak tail; II: flaccid tail; III: II plus hind limb paraparesis (assessed by the animal’s slowness or inability to regain upright status when overturned, splayed limbs when walking); IV: III plus unilateral hind limb paralysis; V: IV plus paralysis of both hind limbs or a forelimb; and VI: moribund, or dead.

All animal studies included in this study have been approved by the McGill Animal Ethics Committee.

Administration of ASO

Mouse XIAP-specific ASO (XIAP ASO; AEG35169) and control antisense oligonucleotides (control ASO1; AEG35187 and control ASO2; AEG35192) were synthesized by Avesca Biotechnology as 4 × 4 mixed backbone 19-mer oligonucleotides, composed of four flanking 2′-O-methyl RNA residues at 3′ and 5′ ends of a central core of phosphorothioate-linked nucleotides, similar to human XIAP-specific ASO AEG35156 (9).

Sequences used were XIAP ASO AEG35169 (targeting the coding region of murine XIAP) (gene symbol birc4, between BIR1 and BIR2), and the control ASO1 (random oligonucleotides) and control ASO2 (containing five mismatched oligonucleotides of nineteen) (Agera Therapeutics). XIAP ASO and control ASOs were administered daily via i.p. injection at a daily dose of 10 mg/kg. ASO treatments were initiated either 5 days before immunization by MOG35–55 for induction of EAE, or at the time of initial individual symptomatic onset of disease (therapeutic model).

Real time analysis of XIAP expression

XIAP mRNA was quantified as previously described (6) by real-time quantitative RT-PCR except that murine-specific XIAP primers and probes were used. XIAP was amplified using primers 5′-CTGAAAAAACACACCGCGTAAC-3′ and 5′-CTAATACCTCAGTATGCCCTTCG-3′ (Integrated DNA Technologies). The TaqMan probe for XIAP was 5′-FAM-AATCGATGATACCATCTTCCAGAATCCTATGGTG-C3′ (Applied Biosystems). Primers and probes for rodent GAPDH were purchased from Applied Biosystems. Total RNA was isolated from whole blood using RNeasy columns combined with DNase I treatment (Qiagen). Twenty to one hundred nanograms of total RNA was reverse transcribed and PCR-amplified using the TaqMan EZ RT-PCR kit (Applied Biosystems). All RT-PCR steps were performed on an ABI Prism 7700 Sequence Detector (Applied Biosystems) and quantified using the cycle threshold method normalized to GAPDH mRNA.

Protein isolation, SDS-PAGE, and Western blotting

Spleens were lysed using radioimmunoprecipitation assay buffer containing 0.015% DTT and complete protease inhibitors (Roche Diagnostic Systems). Cell lysates were centrifuged at 12000 rpm in an Eppendorf microcentrifuge at 4°C for 10 min. The protein in the supernatant was quantified by the Bradford assay (Bio-Rad Laboratories). Twenty micrograms of protein were loaded, separated on a 12% gel by SDS-PAGE, and transferred at 100 volts for 120 min to an Immobilon-P (polyvinylidene fluoride) membrane (Millipore, Bio-Rad Laboratories). Membranes were blocked for 1 h at room temperature in 5% skim milk powder in TBS/0.05% Tween 20 (Sigma-Aldrich). Membranes were probed with anti-XIAP (1/1000; BD Pharmingen) overnight at 4°C in 2% skim milk powder in TBS/0.05% Tween 20. Membranes were washed in TBS/0.05% Tween 20 and reprobed with either an IgG anti-mouse peroxidase, or IgG anti-rabbit peroxidase-labeled Ab (1/100,000; Vector Laboratories). Chemiluminescence was detected using ECL Plus Western Detection Blotting System (Amersham) and scanned using a densitometer with ImageQuant software (Molecular Dynamics). Blots were then stripped (Reblot; Chemicon) and reprobed for the endogenous control protein β-actin (1/10,000; Sigma-Aldrich) to ensure equal protein loading using a peroxidase anti-mouse secondary Ab (1/10,000; Vector Laboratories).

Flow cytometry analysis

Cells were isolated from the CNS and lymphoid tissue as previously described (26). Animals were anesthetized and perfused intracardially with ice-cold PBS. Brain and spinal cord tissue was collected, mechanically dissociated, and centrifuged at 400 × g for 10 min at 4°C. The cell pellet was resuspended in 3% isotonic Percoll (Pharmacia Biotech) and centrifuged at 2800 × g with no brake and moderate acceleration for 20 min at room temperature. Mononuclear cells were collected from the pellet and washed twice in 10% RPMI 1640 (Invitrogen Life Technologies). Cells were blocked with normal rat Ig (Cedarlane) in 2.4G2 (anti-FcγRIII/II) hybridoma supernatant, followed by staining for 15 min at 4°C with the appropriate conjugated mAb. The following mAbs were obtained from BD Pharmingen, anti-CD45-PE/Cy5 (30-F11), anti-CD11b-PE (M1/70), and anti-CD4-FITC (GK1.5). Annexin V-PE staining (BD Pharmingen) was performed after washing cells with Annexin V binding buffer (BD Pharmingen). Samples were analyzed in binding buffer containing 7-aminocinomycin D (7-AAD; BD Pharmingen). Flow cytometry analyses were performed using a FACSscan flow cytometer (BD Biosciences) and analyzed using CellQuest (BD Biosciences).

Histology and immunofluorescence

Mice were anesthetized and perfused intracardially with ice-cold PBS. Tissue samples were collected and embedded in OCT (Electron Microscopy Services). Histochemical and immunofluorescence staining was performed on 10-μm cryostat sections. Frozen sections were fixed in 4% paraformaldehyde, followed by incubation in mouse-on-mouse blocking reagent (Vector Laboratories) for 1 h. For biotinylated primary Abs, sections were also treated with avidin/biotin block (Vector Laboratories). The following primary Abs were used; rat anti-mouse CD11b/Mac-1 (5C6), rat anti-mouse CD45 (IBL-316, Serotec), rat anti-cow GFAP (DAKO), rabbit anti-monosodium glutamate (Chemicon), mouse anti-human myelin basic protein, biotinylated mouse anti-mouse NeuN (Chemicon), and biotinylated rat anti-mouse CD4 (GK1.5, BD Pharmingen). Sections were incubated with primary Ab for 30 min followed by the appropriate fluorescein-conjugated secondary Abs; goat anti-rat Alexa555, streptavidin-Alexa555 (Invitrogen Life Technologies), or goat anti-mouse Cy3 (The Jackson Laboratories) for 1 h at room temperature. Sections were rinsed in 1% paraformaldehyde for 15 min, and further stained for apoptosis using the Fluorescein-Phycoerythrin apoptosis kit (Calbiochem) for TUNEL staining as per the manufacturer’s instructions. Sections were Hoechst (Invitrogen Life Technologies) counterstained and mounted in Prolong-gold mounting medium (Invitrogen Life Technologies). Control sections were incubated with isotype-matched primary Abs. Infiltrates were analyzed on a Leica fluorescence microscope (Leica). Semiqualitative counting of infiltrates was done on H&E stained sections.

Activation induced cell death (AICD)

T cells were purified from lymph nodes (LN) of XIAP ASO or saline treated animals, using Dynal magnetic beads (Dynal). In brief, single cell suspensions were incubated in an Ab mix directed at non-T cells, washed, and separated by depletion of the Ab-labeled cells using depletion dynabeads (Dynal). The negative population was collected, washed, and plated into 96-well plates in the presence or absence of immobilized mouse anti-CD3. Mouse anti-CD3 Ab was purified from I4-2C11 hybridoma supernatant. AICD in CD4 T cells was examined by Annexin V expression. Cells were harvested at 24 h poststimulation, blocked with normal rat Ig (Cedarlane) in 2.4G2 (anti-FcγRIII) hybridoma supernatant, followed by staining for 15 min at 4°C with anti-CD4 FITC (GK1.5; BD Pharmingen). Cells were subsequently washed twice in Annexin V binding buffer (BD Pharmingen), and stained for 15 min in Annexin V-PE with 7-AAD (BD Pharmingen). Cells were analyzed immediately using a FACSscan flow cytometer (BD Biosciences). Dead cells were excluded using 7-AAD.

LN cell proliferation

Proliferation in response to MOG35–55 or purified protein derivative (Cedarlane) was assessed using BrdU. In brief, single cell suspensions were prepared from the draining LN, and 4 × 10^5 cells were cultured cell in 200 μl of RPMI 1640 medium supplemented with 10% bovine growth serum (Sigma-Aldrich), penicillin/streptomycin (Invitrogen), glutamine (Invitrogen), and...
phylactic treatment: most control ASO-treated mice exhibited EAE symptoms measured in therapeutically and prophylactically treated animals. XIAP is elevated in EAE. XIAP ASO reduces XIAP levels.

**FIGURE 1.** XIAP is elevated in EAE. XIAP ASO reduces XIAP levels and prevents or cures EAE. a, EAE was induced by active immunization with MOG35-55/CFA. XIAP mRNA expression in whole blood was examined by qRT-PCR. An ~3.5-fold increase in XIAP mRNA was observed in mice with severe signs of EAE. b, In therapeutic experiments, animals were treated i.p. daily with saline (□), or 10 mg/kg control ASO1 (●), control ASO2 (●), or XIAP ASO AEG35169 (■) from disease onset. Mice treated therapeutically with XIAP ASO dramatically reduce clinical symptoms within 5 days, and chronic symptoms were ameliorated in 5 of 6 mice. Statistically significant differences were observed after day 15 when control-ASO and saline-treated animals exhibited symptoms of relapse (twoway ANOVA, *, p < 0.05). Mean clinical score ± SEM; representative of three experiments. c and d, Incidence of disease (cumulative results) in animals treated prophylactically with 10 mg/kg ASO IP for 5 days before immunization, then daily thereafter. In four experiments where saline treated animals had mild disease (mean clinical score 1.1 ± 1.1) (c), and three experiments with severe disease (mean clinical score 3.9 ± 1.6) (d), control ASO had no impact, and XIAP ASO reduced disease incidence (one-way ANOVA, **, p < 0.001; *, p < 0.05). e and f, XIAP mRNA was measured in therapeutically and prophylactically treated animals. e, Prophylactic treatment: most control ASO treated mice exhibited EAE symptoms (open symbols), whereas most XIAP ASO treated animals were asymptomatic (closed symbols), and showed a significant reduction in the expression of XIAP. f, Therapeutic regime: XIAP ASO-treated animals showed a significant reduction in the expression of XIAP (ANOVA with Newman-Keuls posttests). The p values of <0.05 were considered statistically significant.

**Results**

**Relationship between EAE symptomatology and XIAP expression**

The relationship between EAE symptomatology and XIAP expression was determined in the MOG EAE model. XIAP mRNA levels were assessed by quantitative real-time RT PCR on pooled whole blood from mice exhibiting mild, moderate, or severe EAE symptoms. Increased XIAP expression was found to correlate with the severity of EAE symptoms (Fig. 1a). Mice with severe EAE had greater than 3-fold increases in XIAP mRNA.

**XIAP knockdown cures EAE**

Symptoms of neurological deficit in C57BL/6 mice were normally observed 10–14 days after MOG 35–55 immunization. In the therapeutic variant of the EAE experiment, mice were injected daily with an antisense oligonucleotide, specific for XIAP (XIAP ASO), or various controls, from the first signs of neurological symptoms. Treatment with XIAP ASO rapidly reduced EAE symptoms, such that after 5 days, most mice showed only residual curved tail symptoms (a clinical score of 1) or no symptoms at all (Fig. 1b). Continued XIAP ASO treatment led to a statistically significant reduction (five of six animals) in the subsequent exacerbations of symptoms observed between days 16–25 (two-way ANOVA). By comparison, all animals treated with a randomized control oligonucleotide, control ASO1 (n = 6), or a mismatch control sequence, control ASO2 (n = 6), showed exacerbations of EAE symptoms, as did nine of ten animals in the saline-treated group (Fig. 1b).
There was a trend toward more severe disease in the control oligonucleotide-control treated animals compared with the saline treatment group, but this was only statistically significant at day 3 of treatment. Although this may reflect a nonspecific effect of oligonucleotides, the fact that it exacerbated disease strengthens the case for the protective effects of ASO-XIAP. The effect of cessation of ASO treatment was not considered in this study, although it is probable that disease would re-emerge in the absence of the therapy.

**XIAP knockdown prevents EAE**

In a second treatment paradigm, animals were subjected to a 5-day prophylactic antisense treatment before MOG immunization, followed by daily treatment for 25 days. Several independent experiments were conducted using this regimen. Analysis of the cumulative results from experiments in which control animals developed only mild disease (mean clinical score of 1.1 ± 1.1), showed that the XIAP ASO-treated groups had an EAE incidence of 8.8 ± 0.6% (n = 34) and a mean clinical score of 0.1 ± 0.1 (Fig. 1c). Saline and control ASO1-treated groups showed EAE incidences of 70.4 ± 4.6% (n = 54) and 55.4 ± 7.6% (n = 27), respectively (Fig. 1c). In prophylactic experiments where saline-treated animals showed severe disease, exhibiting a mean clinical score of 3.9 ± 1.6 and an incidence of 97.9 ± 2.0% (n = 28), the clinical effect of XIAP ASO was also significant (Fig. 1d). In this case, the mean severity of disease in XIAP ASO-treated animals was 0.8 ± 1.6, and the incidence of disease was 21.9 ± 9.0% (n = 25). The random control ASO1 had no significant effect on disease incidence (67.4 ± 21.3%, n = 28) although severity of disease (1.2 ± 1.5) was reduced. In MOG35–55-immunized mice prophylactically treated with XIAP ASO, a significant knockdown of XIAP expression was observed in peripheral blood (Fig. 1e). XIAP ASO-specific knockdown was also observed in mice treated therapeutically with the XIAP ASO AEG35169, after onset of EAE symptoms (Fig. 1f), indicating that low XIAP levels observed following prophylactic XIAP ASO dosing (Fig. 1e) were likely due to the treatment rather than a consequence of failure to exhibit EAE. By contrast, neither of the control oligonucleotides caused a reduction in XIAP-RNA expression in blood when MOG35–55-immunized mice were dosed by either regimen (Figs. 1, e and f). It was of note that the only animal in the prophylactically treated XIAP-ASO group that developed EAE, did not achieve XIAP knockdown (Fig. 1e). Western blot analysis of protein extracted from the spleens of XIAP ASO-treated and control ASO-treated animals confirms the biological relevance of the knockdown observed by PCR. Significant knockdown in XIAP protein levels was only observed in XIAP-treated animals (Fig. 1g and h). Knockdown in XIAP mRNA expression was also observed in the liver of XIAP ASO-treated animals, but with no concurrent increase in hepatocyte apoptosis, as assessed by TUNEL staining (data not shown).

Amelioration of EAE symptoms was not due to a Th1/Th2 shift in treated animals, as measured by RNase protection assays of cytokine expression in spleen tissues (data not shown). Moreover RNase protection assays of cytokine and chemokine expression in the CNS revealed dramatic reductions in the expression of inflammatory responses in both prophylactically and therapeutically treated animals (Fig. 2). Expression of interleukins IL-1ra, IL-1β, IL-1α, and IL-6; chemokines TCA-3/CCL1, MCP-1/CCL2, IP-10/CXCL10, MIP-1α/CCL3, MIP-1β/CCL4, and MIP-2/CXCL1; and RANTES/CCL5 were all dramatically reduced in the CNS of XIAP ASO-treated animals in both the prophylactic and therapeutic regime. No significant differences were observed in levels of IL-10, IL-12, IFN-γ, IL-18, or lymphotactin (Fig. 2). The efficacy of the therapeutic treatment regime indicated that XIAP-ASO was acting at the level of the effector response rather than suppressing the induction of immunity. The dampened inflammatory response in the CNS further reinforces a role for XIAP ASO in regulating the effector response and progression of the disease.

**Reduced T cells in the CNS of ASO XIAP-treated animals**

Flow cytometry was used to analyze the cellular phenotype of the CNS infiltrate that is a normal consequence of MOG35–55 immunization. Mononuclear cells were isolated from the CNS of individual animals treated with XIAP ASO or control ASO at the time of peak disease in the control animals in prophylactically treated groups and at end of treatment (day 25) in the therapeutic treatment groups. Levels of CD45 expression were used to discriminate between blood-derived cells (CD45<sup>high</sup>) and resident microglial cells (CD45<sup>low</sup>), which also express many myeloid markers (27). Expression of the myeloid marker CD11b/Mac-1 was used to discriminate T cells (CD45<sup>high</sup>CD11b<sup>high</sup>) from macrophages and neutrophils (CD45<sup>high</sup>CD11b<sup>low</sup>). Infiltrating cells were observed in the majority of animals irrespective of the treatment regime, clinical score, or the time of sampling. However, proportions of T cells in XIAP ASO-treated animals decreased compared with the saline-treated animals (Figs. 3, a and b). There was a significant reduction...
in the total number of infiltrating T cells in XIAP ASO-treated animals (either prophylactic or therapeutic) compared with the control ASO or saline-treated groups (Fig. 3, c and e). There were slight reductions in the numbers of macrophages and neutrophils infiltrating the CNS of prophylactically XIAP ASO-treated animals, but this was not significant (Fig. 3d). This dramatic reduction in T cells within the CNS coincides with the lack of symptoms in the case of prophylactic treatment and the significant reduction in clinical symptoms in the therapeutically XIAP ASO-treated animals.

Apoptosis of effector T cells in ASO XIAP-treated CNS

The lack of clinical symptoms in the XIAP ASO-treated animals reflected the apoptosis of activated effector T cells. Tissue sections taken at the time of peak disease in the control mice were stained for the T cell marker CD4 and double TUNEL labeled to identify apoptotic cells. CD4+/TUNEL−/TdT+ cells were identified as cells stained with a CD4 surface stain (red) and TdT+ nuclei (green) (Fig. 4a). Cells with cytoplasmic TdT, presumably phagocytic macrophages, were not counted. Reduced numbers of infiltrating T cells (red) could be readily observed in the XIAP ASO-treated animals (Fig. 4b) as compared with saline controls (Fig. 4c). The total number of CD4+ cells per infiltrate was significantly reduced in XIAP ASO-treated animals (Fig. 4d). Strikingly, the proportion of these CD4+ cells which were TdT+, was significantly elevated (Fig. 4e). These histology results were confirmed by FACS analysis of CNS infiltrates. Although only a limited number of T cells were isolated from XIAP-treated animals, a significantly higher proportion of these cells express Annexin V on their surface as compared with control and saline treatment groups (Fig. 4f). The proportion of cells identified as apoptotic in control and saline-treated groups was higher when analyzed by Annexin V staining as compared with TdT. This may be a result of trauma to the cells...
during the extraction procedure, or the timing of the analysis, as samples were analyzed at peak disease.

Proapoptotic effect is restricted to T cells
The absence of severe or sustained clinical symptoms in the XIAP ASO-treated animals is one indication that there were no deleterious effects on neurons or oligodendrocytes, and suggests that the proapoptotic effect is restricted to the effector T cells. To confirm this, tissue sections were double stained by TUNEL and neural cell markers. Using a combination of mAbs for infiltrate (CD45, CD4, and CD11b/Mac-1), infiltrate/endothelium (ICAM-1), and endothelium (Laminin), we showed that the vast majority of TUNEL⁺ cells were restricted to perivascular infiltrates in white matter tracts of the spinal cord (Fig. 5 and data not shown). In addition TUNEL⁺ cells in white-matter of XIAP ASO-treated animals did not colocalize with the mature oligodendrocyte marker CC1 (arrowheads, Fig. 5a). Further analysis using the marker NeuN to identify neuronal cell bodies illustrated that the rare TdT⁺ cells found in the gray matter were not neurons (Fig. 5, b and c).

Perivascular and meningeal infiltrates were observed in the majority of animals, irrespective of the treatment regime, clinical score, or the time of sampling, although in XIAP ASO-treated animals, infiltrates were markedly smaller (Fig. 6, a–d) as compared with control-ASO (Fig. 6, e and f) or saline-treated animals (Fig. 6, g and h). These infiltrates contained both CD4⁺ T cells (Fig. 6, a, c, e, and g) and CD11b⁺ cells (Fig. 6, b, d, f, and h). No significant differences were observed in either the degree of dissemination of the infiltrate into the parenchyma, or the regional location of infiltrates within the CNS. Counts of infiltrate foci in tissues at peak or equivalent time points showed significant reduction in XIAP ASO-treated animals, compared with saline-treated controls (Fig. 6i). Although lower numbers of infiltrate foci were observed in control ASO1- and control ASO2-treated animals as

\[ \text{FIGURE 5.} \text{ Apoptosis is restricted to infiltrating leukocytes.} \text{ a, In XIAP ASO AEG35169 prophylactically treated animals at the time of peak disease in control treated animals, the majority of TUNEL⁺ dying cells are restricted to the areas of the infiltrate in the white matter tracts of the spinal cord, whereas CNS resident cells are not affected. Oligodendrocytes stained with CC1 (arrowheads; red) do not colocalize with TUNEL⁺ dying cells (green), b and c, Double staining of neuronal cell bodies with NeuN (red) and TUNEL (green) illustrates the rare green cells present in the gray matter are not neurons. Line denotes the border between white and gray matter. Scale bar, 25 \mu m.} \]

\[ \text{FIGURE 6.} \text{ Immunohistology of infiltration. Infiltration of CD4⁺ T cells (red, a, c, e, and g) and CD11b/Mac-1⁺ cells (red, b, d, f, and h) in prophylactically treated XIAP ASO AEG35169 animals at peak disease (a and b), therapeutically treated XIAP ASO AEG35169 at 25 days (c and d), control-ASO1 treated animals (e and f), and saline-treated (g and h) animals at peak disease. T cells and macrophages can be observed in the meningeal and perivascular areas of white matter tracts of the spinal cord. Scale bar, 100 \mu m. Reduced areas of infiltrate were observed in both prophylactically and therapeutically treated XIAP ASO AEG35169 animals. i, Semi-quantitative analysis of sections from saline ( ), prophylactic control ASO1 ( ), and prophylactic XIAP ASO AEG35169-treated animals ( ) show significant reduction in total infiltration with ASO AEG35169 treatment. Open symbols illustrate animals with clinical symptoms. Sixteen sections/animal throughout the length of the spinal cord were examined; results represent the total infiltrate. Statistical significance was analyzed by one-way ANOVA; *, p < 0.05.} \]
Increased susceptibility to AICD induced by ASO XIAP

To further dissect the effect of XIAP knockdown, we analyzed AICD in response to TCR activation in vitro. LN T cells derived from XIAP ASO treated and control animals were purified by negative selection and cultured in the presence or absence of plate-bound anti-CD3 for 12–24 h. The percentage of AICD was measured by Annexin V labeling, which detects cells at an early stage of apoptosis. As shown in Fig. 7, a significant increase in apoptotic cell death was observed in the activated CD4⁺ T cells derived from XIAP ASO-treated animals. This confirmed that XIAP ASO conferred susceptibility to Ag-induced activation and apoptosis of effector T cells, is likely the basis for its therapeutic effect.

Discussion

We have shown that CNS-infiltrating effector T cells are more prone to apoptosis when they have been exposed to XIAP antisense. This leads to a loss of T cell effectors within the CNS, and an aborted inflammatory response, which prevents or cures EAE, with no concurrent adverse effects on neural tissue. These findings support the principle that manipulation of effector T cell predisposition to apoptosis is an effective route for control of immunologic diseases.

A pro- vs anti-apoptotic imbalance has been proposed to play a significant role in disease pathogenesis (15, 28). Defective T cell apoptosis has been attributed to many factors including elevated soluble CD95 (29), over expression of the caspase 8 inhibitor, FLIP in T cells (30), increased IAP (18, 31), and Bcl-XL (32). Diminished autoimmune T cell responsiveness to apoptotic triggers may be due to differential induction of proapoptotic stimuli, reflecting variations in costimulator environment or affinity for MHC⁺ Ag, or due to failure to mount the caspase activation and/or execution sequence due to defects in pathways such as Fas or TRAIL (14, 33, 34).

XIAP is a key regulator of receptor-mediated death (6). Genetic lack of XIAP leads to a lymphoproliferative syndrome (35). The elevated levels of XIAP detected in activated lymphocytes of human MS patients (19) may protect these T cells from apoptosis and so promote their autoimmune effector function. A similar mechanism may operate in T cells from Lck XIAP transgenic mice which develop T cell tumors. Cells derived from hematological tumors in these mice maintain a heightened apoptotic threshold (36). Alternative targeting of XIAP has obvious clinical potential. We have shown in EAE, that elevations in XIAP also occur, and that this diminishes the apoptotic potential of the autoreactive T cells. One would predict that as the need for anti-apoptotic protection decreases, T cells might reduce XIAP levels appropriately. Reducing XIAP expression with systemic antisense treatment may also serve the same purpose.

The fact that antisense XIAP operates independently of the Ag specificity of disease-causing T cells has potential advantages, allowing it to be used in a variety of situations. For instance, the Ags that induce MS are unknown, although many myelin epitope specificities have been implicated. XIAP inhibition would be effective for all of them. Deleterious consequences of other inflammatory responses could be similarly controlled. Components of the innate immune response such as macrophages are not affected because apoptosis is not the primary mechanism for regulation of macrophage infiltration (37). Finally, potentially protective T cell specificities, not engaged at time of therapy, are not affected. Effector apoptosis therapy could therefore be applied to a broad range of patients without regard for repertoire differences and likely would be effective against pathologies that involve Ag-specific effectors.

The fact that Ag-independent arms of the immune response, such as macrophage activation, are not affected by XIAP knockdown, and specifically that entry of such cells to the CNS is not inhibited, as we have shown, means that innate host-protective immunity is retained.

XIAP is a key regulator of apoptosis and as such is an attractive target for inducing increased cell death, most likely through enhanced sensitization to extrinsic cell death factors (38). Antisense oligonucleotides hold significant promise for the treatment of disease. Sequence specificity greatly limits off-target toxicities and current chemical designs have greatly increased stability in vivo (21). The XIAP ASO AEG35156, a second-generation mixed backbone antisense oligonucleotide specific for human XIAP is currently the subject of multiple Phase I trials (39). Effector T cells, whose natural fate is apoptosis, may require only partial XIAP down-regulation to be rendered susceptible to apoptotic triggers after they invade the CNS. Indeed, our ex vivo studies show their heightened susceptibility to activation-induced-apoptosis. Thus, the highly primed nature of these cells presents a therapeutic window wherein they can be predisposed to apoptosis without affecting apoptosis of other cells such as neurons, or oligodendrocytes, which are the prime targets in MS.

Given the remarkable feed-forward resulting from the release of activated caspases during apoptosis, even slight reductions in anti-apoptotic IAP signals may well convey enhanced sensitivity to those cells which are primed for apoptosis, while not affecting normal cells. These findings support the theoretical view that an elevated apoptotic threshold is maintained by elevated XIAP in cells that are otherwise primed for apoptosis.

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Disclosures

The authors have no financial conflict of interest.
References


