Defective Fas expression exacerbates neurotoxicity in a model of Parkinson's disease

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Fas (CD95), a member of the tumor necrosis factor–receptor superfamily, has been studied extensively as a death-inducing receptor in the immune system. However, Fas is also widely expressed in a number of other tissues, including in neurons. Here, we report that defects in the Fas/Fas ligand system unexpectedly render mice highly susceptible to neural degeneration in a model of Parkinson's disease. We found that Fas-deficient lymphoproliferative mice develop a dramatic phenotype resembling clinical Parkinson's disease, characterized by extensive nigrostriatal degeneration accompanied by tremor, hypokinesia, and loss of motor coordination, when treated with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) at a dose that causes no neural degeneration or behavioral impairment in WT mice. Mice with generalized lymphoproliferative disease, which express a mutated Fas ligand, display an intermediate phenotype between that of lymphoproliferative and WT mice. Moreover, Fas engagement directly protects neuronal cells from MPTP/1-methyl-4-phenylpyridinium ion toxicity in vitro. Our data show that decreased Fas expression renders dopaminergic neurons highly susceptible to degeneration in response to a Parkinson-causing neurotoxin. These findings constitute the first evidence for a neuroprotective role for Fas in vivo.

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Fas is commonly categorized as a death receptor because of its well-defined role in apoptosis (1). It is expressed throughout the central nervous system, including in glia and neurons (2), and induces neuronal apoptosis under certain conditions, such as in models of stroke and amyotrophic lateral sclerosis (3–5). In this report, we investigated the role of Fas signaling in a mouse model of Parkinson's disease (PD). PD is a chronic and debilitating neurodegenerative disorder, characterized by degeneration of the midbrain dopaminergic neurons of the substantia nigra pars compacta (SN), resulting in the hallmark symptoms of the disease, namely tremor, bradykinesia, rigidity, and postural instability. The etiology of PD is unknown, and in up to 95% of cases there is no identified genetic linkage (6). Environmental factors, such as neurotoxic pesticides, have been implicated in disease pathogenesis. Exposure to the dopaminergic toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) provides a well-established model of PD in rodents and primates and has been shown to cause PD in humans (6). MPTP crosses the blood–brain barrier where it is metabolized to its active form, the 1-methyl-4-phenylpyridinium ion (MPP^+), which is concentrated selectively in dopaminergic neurons by the dopamine transporter, resulting in dopaminergic neuronal death.

In PD patients, membrane-bound Fas and Fas ligand (FasL) expression are reduced in the SN (7). Concomitantly, soluble Fas, which acts as a decoy receptor and blocks the binding of FasL to Fas, is elevated in PD (8, 9). Thus, Fas signaling in PD patients may be diminished by reduced cell surface Fas expression and by the presence of soluble Fas. Apoptosis is believed to be a factor in the neurodegeneration of PD (10), but in vitro models indicate that caspase-8, the upstream caspase activated during Fas-mediated apoptosis, is not involved, suggesting that Fas may not be the principal death effector in PD neurodegeneration (11).

The online version of this article contains supplemental material.

We used mice bearing mutations in the Fas/FasL system to determine directly the role of Fas signaling in the MPTP model of PD. We demonstrate here that reduced Fas expression dramatically increases neuronal susceptibility to MPTP toxicity in vivo, strongly supporting a neuroprotective role for Fas.

RESULTS AND DISCUSSION

Fas deficiency results in markedly increased susceptibility to MPTP-induced dopaminergic neuron degeneration

We compared the MPTP susceptibility of WT C57BL/6 (B6), Fas-deficient lymphoproliferative (*lpr*), and FasL-mutated generalized lymphoproliferative (*gld)* mice that share the B6 genetic background. *Lpr* mice express low to absent cell surface Fas because of the insertion of a transposable element within a Fas intron (12). *Gld* mice have a point mutation in the extracellular domain of FasL that decreases the affinity of the Fas/FasL interaction, resulting in reduced signaling through Fas (13, 14). We treated mice with a subacute toxicity regimen of MPTP consisting of an injection of 25 mg/kg/d for 5 consecutive d and quantified tyrosine hydroxylase positive (TH^+) neurons as a marker for dopaminergic neurons in the midbrain (SN and ventral tegmental area, VTA) 4 d after the final injection of MPTP. Consistent with previous findings (15), WT mice treated with this subacute regimen of MPTP exhibited only a marginal, nonsignificant loss of midbrain dopaminergic neurons at this time point (Fig. 1, A–C). In contrast, we observed a dramatic loss of dopaminergic neurons in Fas-deficient mice. We found that the SN and VTA of MPTP-treated *lpr* mice showed a 58% ($P < 0.001$) and 40% ($P < 0.05$) loss of dopaminergic neurons, respectively, compared with saline-treated *lpr* mice. MPTP-treated *gld* mice lost 26% ($P < 0.05$) of the dopaminergic neurons in the SN compared with saline-treated *gld* mice (Fig. 1, A–C). The pretreatment number of TH^+ neurons was not significantly different in *lpr*, *gld*, and WT midbrains (Fig. 1, B–C). The depletion of neuron cell bodies in the SN of MPTP-treated *lpr* mice was confirmed in cresyl violet–stained sections (unpublished data). We also observed a striking decrease in dopaminergic innervation to the striatum in MPTP-treated *lpr* mice and to a lesser extent in *gld* mice (Fig. 1 D). In fact, the loss of terminals in the caudate putamen of the striatum and the sparing of the olfactory tubercle and nucleus accumbens in MPTP-treated *lpr* mice resemble the pattern of terminal loss seen in the striatum of PD patients. The increased susceptibility of *lpr* mice to MPTP occurs despite lower levels of MPP^+ accumulation in the striatum than WT B6 mice, as determined by HPLC 4 h after the third dose of MPTP (B6: 3.29 ± 0.15 μg/g tissue; *lpr*: 2.29 ± 0.12 μg/g tissue; *gld*: 2.13 ± 0.37 μg/g tissue). Increased neurodegeneration in the presence of decreased levels of bioavailable MPP^+ further emphasizes the enhanced sensitivity to neurodegeneration in the absence of Fas. Thus, Fas-deficient *lpr* mice treated with MPTP display a dramatic degeneration of the nigrostriatal system. *Gld* mice, which have a partial Fas-signaling deficit (14), demonstrate an intermediate degree of neuronal loss.

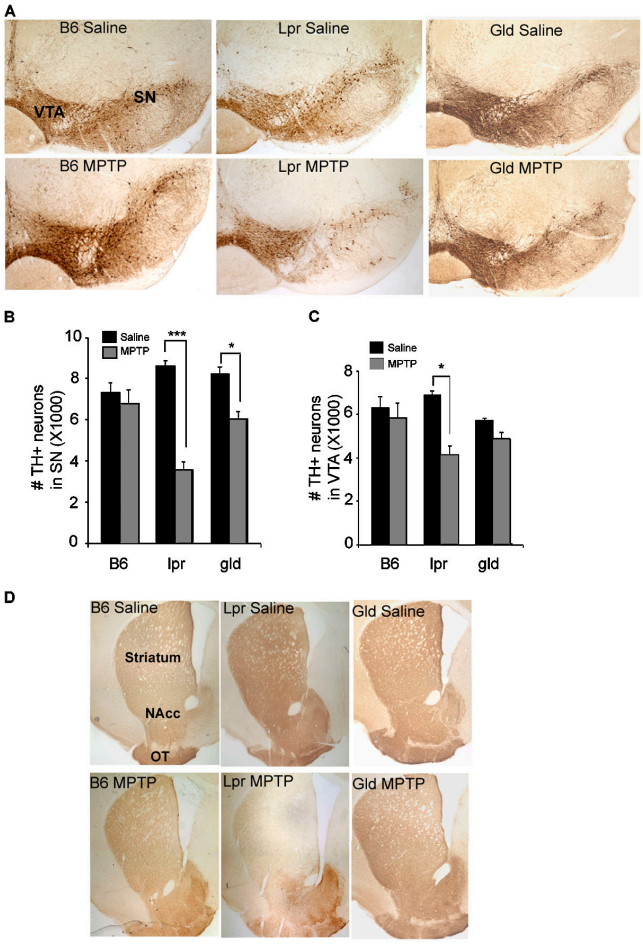

Figure 1. Massive loss of dopaminergic neurons and striatal projections in MPTP-treated Fas-deficient mice. (A) TH-stained sections through the midbrain (SN and VTA) in MPTP- or saline-treated WT, *lpr*, and *gld* mice. (B, C) The number of TH^+ neurons in the SN (B) and VTA (C) was determined by unbiased stereology ($n = 4$; *, $P < 0.05$; ***, $P < 0.001$; error bars show SE). (D) TH-stained sections through the striatum in saline- or MPTP-treated WT, *lpr*, and *gld* mice. No loss of staining is observed in WT striata, but extensive depletion of SN projections to the caudate putamen with relative sparing of the nucleus accumbens and olfactory tubercle is seen in *lpr* mice.

Fas deficiency results in tremor, hypokinesia, and decreased motor coordination after MPTP treatment

WT mice did not exhibit significant spontaneous behavioral changes during or after the MPTP treatment course. In sharp contrast, we found that Fas-deficient *lpr* mice displayed marked hypokinesia (Fig. 2 A) and developed a tremor after the second to third dose of MPTP (Videos 1–3, available at http://www.jem.org/cgi/content/full/jem.20050163/DC1). Over four independent experiments, 78% of the *lpr* mice ($n = 32$) became immobile during the first 4 d after the initiation of MPTP treatment, although they gradually recovered spontaneous mobility (Fig. 2 B). WT mice maintained normal activity levels throughout the treatment course (Fig. 2, A and B), and *gld* mice displayed a behavioral

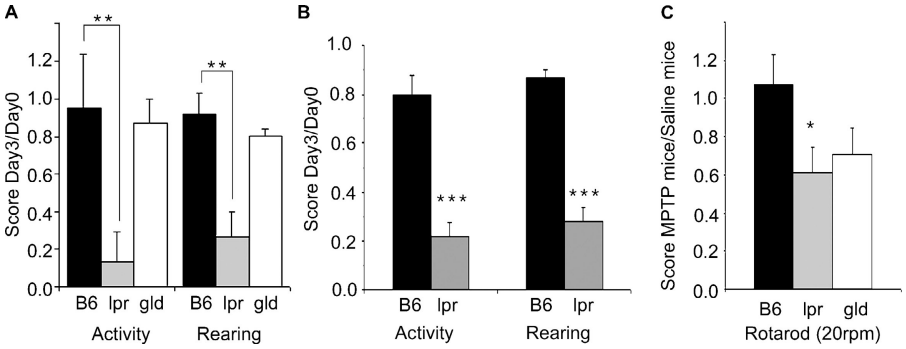

Figure 2. *Lpr* mice become profoundly hypokinetic after exposure to MPTP. (A) Total activity and rearing are shown for WT, *lpr*, and *gld* mice (same mice as in Fig. 1). Scores were generated as the ratio of pretreatment to posttreatment activity for each mouse. Activity was measured 20 h after the second injection of MPTP to avoid the acute effects of the toxin. **, $P < 0.01$. (B) Pooled behavioral analysis over four experiments is shown (B6: $n = 18$; *lpr*: $n = 32$). ***, $P < 0.001$. (C) Rotarod scores were calculated as the ratio of performance of MPTP-treated to saline-treated mice 3 d after the final injection of MPTP. *, $P < 0.05$.

phenotype more closely resembling that of WT mice, correlating with their less severe neuronal loss (Fig. 2 A).

Over the 5 d of MPTP treatments, the *lpr* mice were too impaired for assessment of coordination. 3 d after the final MPTP injection, we found no impairment in WT mice, consistent with previous work (15). However, we observed that *lpr* mice had impaired coordination by Rotarod testing, achieving only 39% ($P < 0.05$) of the score of their WT counterparts (Fig. 2 C). *Gld* mice seemed to be slightly impaired, but this decrease did not reach statistical significance. Thus, Fas-deficient mice became severely hypokinetic, developed tremor, and displayed decreased coordination after exposure to MPTP at a dose that causes no spontaneous behavioral deficits in WT mice.

These results suggest that Fas provides a neuroprotective signal that is missing in *lpr* mice and is reduced in *gld* mice. This putative Fas-induced neuroprotective signal is probably ligand dependent, because *gld* mice, like *lpr* mice, displayed increased susceptibility to neuronal loss after MPTP administration, although degeneration was less extensive in *gld* mice than in *lpr* mice. The residual signal transmitted through Fas in *gld* mice (14) might explain the less severe degenerative and behavioral phenotype seen in *gld* mice as compared with *lpr* mice.

Fas engagement is protective against MPTP/MPP$^+$ neurotoxicity in vitro, and this neuroprotection is independent of caspase-8 activation

To determine whether Fas engagement is directly protective against MPTP toxicity in dopaminergic neurons, we treated cultured midbrain neurons with a FasL construct to induce Fas signaling, followed by exposure to MPTP. Treatment with FasL protected primary TH$^+$ neurons from MPTP toxicity (Fig. 3 A). We repeated this experiment using the Fas-positive neuroblastoma cell line SH-SY5Y to determine whether Fas-induced neuroprotection operated independently of glia. We found that administration of FasL significantly reduced MPP$^+$ toxicity (Fig. 3 B). Fas-mediated protection did not occur in a Fas-negative subline of SH-SY5Y cells (Fig. 3 B). To determine whether the neuroprotective effect of Fas required caspase-8 activation, we used a specific cell permeant caspase-8 inhibitor, IETD-fmk, which effectively protects Jurkat T cells from FasL-induced apoptosis (Fig. 3 C). Unlike Fas-mediated apoptosis, Fas-mediated neuroprotection was not blocked by IETD-fmk (Fig. 3 D). Thus, Fas engagement protected dopaminergic neurons from MPTP/MPP$^+$ toxicity even in the absence of glia and independently of caspase-8 activation.

Fas-mediated neuroprotection in vivo is independent of the Fas death domain

Our in vitro data suggested that Fas-mediated neuroprotection may be independent of the Fas death domain (DD), because the DD serves to anchor and activate caspase-8 via the adaptor protein Fas-associated DD. We tested this hypothesis in two strains of mice bearing disruptions in their Fas DD. Mice bearing an engineered disruption of the Fas DD (16) commonly are referred to as "Fas null," because Fas-induced death is abolished in these mice. However, the mice express a truncated Fas molecule lacking exon 9, which encodes most of the DD, but retain the remainder of the intracellular Fas domains as well as Fas expression at the cell surface, unlike *lpr* mice, which express no detectable Fas by flow cytometry (Fig. 4 A). These Fas exon 9 KO mice are resistant to MPTP (Fig. 4 A and reference 17), in contrast to the dramatic susceptibility of *lpr* mice, as shown by striatal dopamine and dopamine metabolite levels. These mice show no significant differences in MPP$^+$ levels (Exon 9 KO: 3.48 ± 0.34 μg/g; B6: 3.29 ± 0.15 μg/g). We propose that this difference in susceptibility between *lpr* and exon 9 KO mice is based on Fas expression levels (Fig. 4 A), with Fas expression being neuroprotective.

An analogous, naturally occurring point mutation, *lpr.cg*, causes local unfolding of the DD and likewise abolishes Fas-induced apoptosis, although *lpr.cg* mice express normal levels of cell surface Fas and can mediate nonapop-

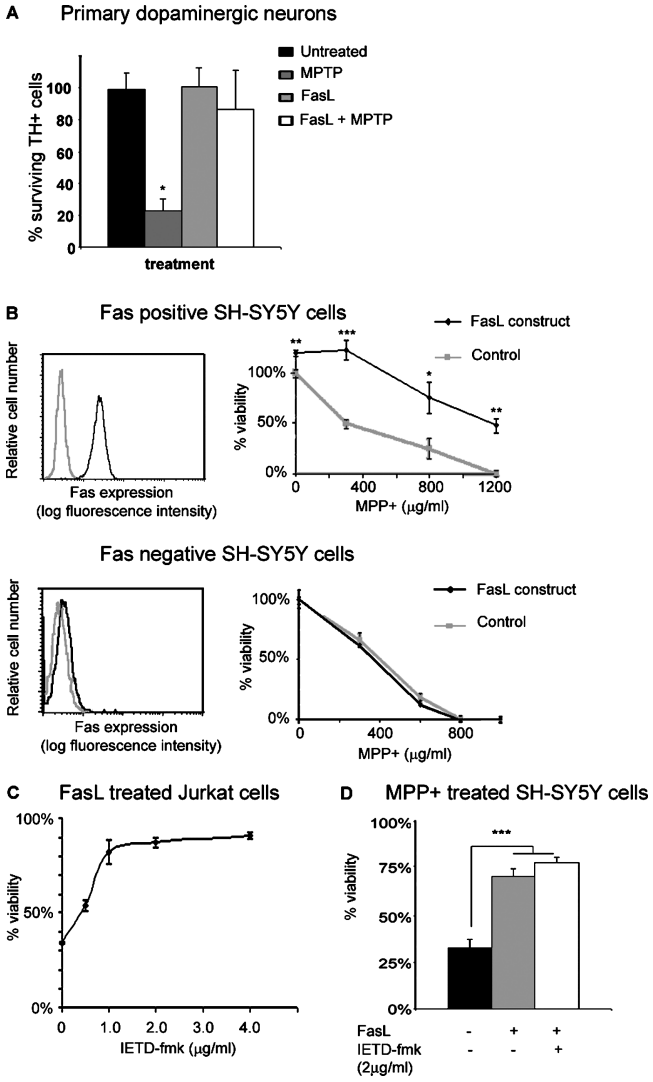

Figure 3. Fas-induced neuroprotection is independent of caspase-8 activation. (A) Primary dopaminergic neurons were treated as shown with FasL and/or 50 μM MPTP. Untreated cells are defined as having 100% viability. (B) Fas-positive and -negative SH-SY5Y cells (as assessed by flow cytometry) were treated with FasL (black line) or PBS (gray line) 30 min before MPP^+ exposure. 100% viability represents untreated SH-SY5Y cells. (C) Jurkat cells were killed by FasL, and this cell death was blocked by the caspase-8 inhibitor IETD-fmk. (D) SH-SY5Y cells were treated with IETD-fmk 1 h before addition of FasL and/or 800 μg/ml MPP^+ to the culture. Error bars show SE.

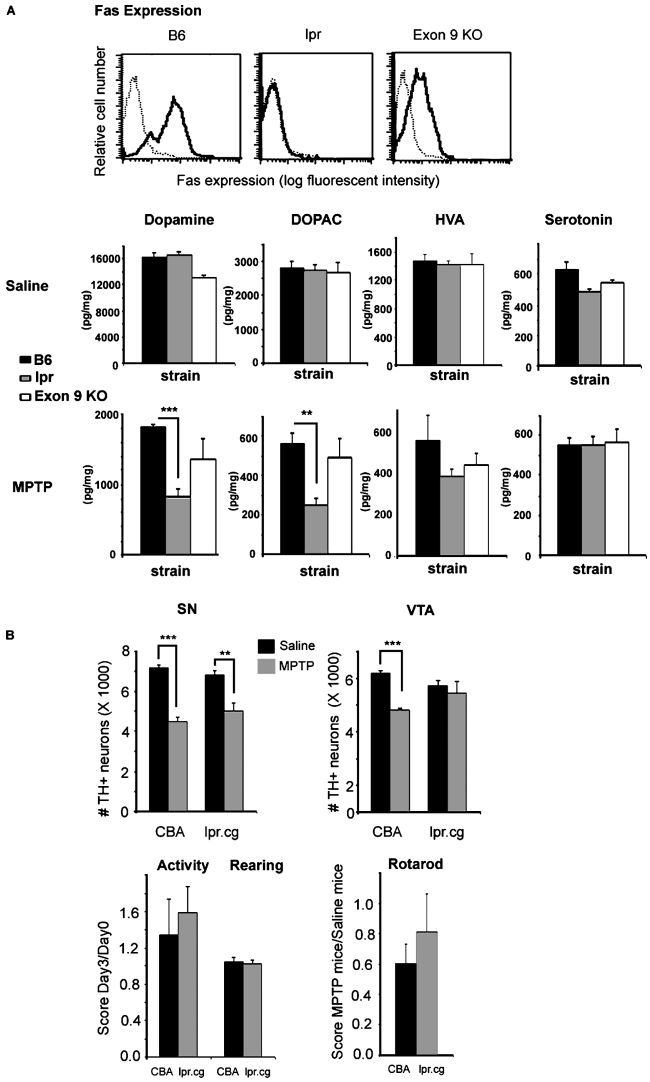

Figure 4. Fas-mediated neuroprotection in vivo is independent of the Fas DD. (A) Exon 9 KO mice have a deleted DD and are no more susceptible to MPTP than are WT mice. *Lpr* mice (MPTP-susceptible) do not express detectable Fas, whereas WT and exon 9 KO mice express Fas as determined by flow cytometry (Fas expression, solid line; background staining, dotted lines). MPTP-treated *lpr* mice show reduced striatal dopamine, dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), as determined by HPLC, compared with B6 and exon 9 KO mice. Serotonin levels were included as a control and were not affected by MPTP. (B) *Lpr.cg* mice have a disrupted DD and are no more susceptible to MPTP than WT CBA mice, as shown by TH^+ neuron counts (saline-treated, black bars; MPTP, gray bars; $n = 3$) and behavior (CBA, black bars; *lpr.cg*, gray bars; $n = 4$–6). Methodology was as described in Figs. 1 and 2.

totic signaling (18). The *lpr.cg* mutation is available only on the CBA background; therefore, *lpr.cg* mice cannot be compared directly with *lpr*, Fas exon 9 KO, and *gld* mice, all of which are on the B6 background. Therefore, we compared MPTP susceptibility of *lpr.cg* mice with that of their WT CBA counterparts. We found no significant difference in TH^+ neuron loss in the SN of *lpr.cg* versus WT CBA mice (Fig. 4 B); in fact, we observed a statistically significant protection in the VTA of *lpr.cg* compared with CBA mice (Fig. 4 B). Behaviorally, both Fas exon 9 KO and *lpr.cg* mice were resistant to MPTP administration (reference 17 and Fig. 4 B, respectively). Together, these data indicate that a functional DD is not required for Fas-mediated neuroprotection. The observation that Fas exon 9 KO (17) and *lpr.cg* mice are slightly more resistant to MPTP than are WT mice suggests that Fas neuroprotective signaling in the absence of Fas apoptotic signaling may confer

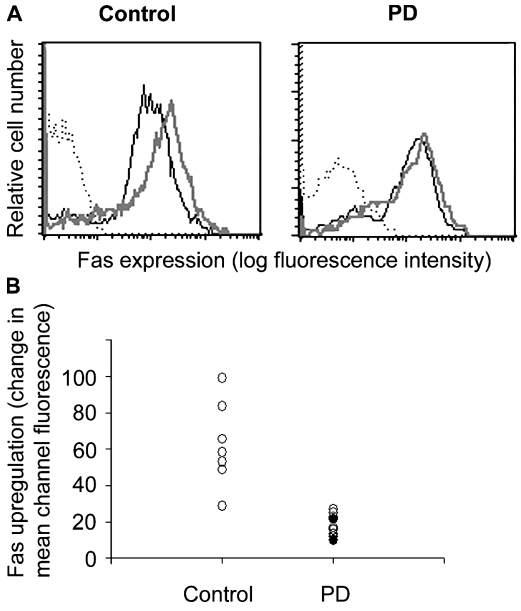

Figure 5. Fas up-regulation is decreased in patients who have PD. (A) Representative flow cytometry histograms show that cell surface Fas expression is up-regulated by activation on PBTs from control subjects but not on PBTs from patients who have PD. Histograms show background staining (dotted lines), Fas expression on unstimulated T cells (black lines) and Fas expression on activated T cells (thick gray lines). (B) Fas up-regulation on T cells, calculated as the difference in mean Fas fluorescence between unstimulated and activated T cells (as shown in 5 A), is shown for control subjects and patients who have PD ($P < 0.001$). Filled circles represent the two PD patients not taking medication. Proliferation (% blast, 12.0 $\pm$ 2.5 for controls; 13.7 $\pm$ 4.9 for PD patients) and viability (97 $\pm$ 1 live cells for controls, 95 $\pm$ 2 live cells for PD patients) were equivalent in the T cells from control and PD patients.

additional protection from MPTP toxicity. The abnormal immune system, which is characteristic of mice lacking Fas-induced death, is present in Fas exon 9 KO, *gld*, *lpr.cg*, and *lpr* mice, demonstrating that the immune abnormality in itself does not alter neuronal susceptibility to MPTP toxicity, because *lpr.cg* and exon 9 KO mice are protected, whereas *lpr* mice become dramatically more susceptible.

Fas is up-regulated in the midbrain in response to MPTP (17) and, indeed, is up-regulated by multiple types of injuries or stress to the central nervous system (2). We propose that Fas up-regulation may represent an adaptive response to stress, resulting in neuroprotection when the cell is salvageable and neuronal apoptosis when the damage is too severe for the cell to be rescued. We have recently shown that Fas engagement can mediate neurite outgrowth via activation of the extracellular-signal regulated kinase (ERK) pathway, independent of caspase-8 (18). Fas engagement similarly can activate ERK in neural stem cells (19). Furthermore, it has been demonstrated previously that ERK phosphorylation can mediate a protective stress response in neurons (20), and there is growing evidence that neuroprotective kinase pathways, when disrupted, may lead to genetically determined PD in humans (21). Thus, Fas expressed by stressed neurons may provide protective signals via protein kinase pathways that, when disrupted, lead to increased susceptibility to PD.

PBLs from PD patients show decreased Fas up-regulation upon activation

Because of the extreme susceptibility to toxin-induced PD demonstrated by mice with reduced Fas expression, we investigated Fas expression in patients diagnosed as having idiopathic PD. We obtained blood samples from patients who had PD and from controls who did not have PD as a simple and minimally invasive means to measure Fas expression. We found that baseline Fas expression in PBLs from patients who had PD did not differ from that in control subjects who did not have PD. However, when we stimulated the peripheral blood T cells (PBTs) with mitogen to induce Fas up-regulation, we found a highly significant deficit ($P < 0.001$) in the ability of PBTs from PD patients to up-regulate Fas (Fig. 5). Thus, patients who have PD show an impairment of Fas up-regulation, at least in PBTs.

Although it is possible that the medication prescribed for PD may influence Fas regulation, two of the patients included in our study were not taking any medication, and both of these individuals displayed very low levels of Fas up-regulation, similar to levels in treated patients who had PD. Furthermore, there was no correlation between level of Fas up-regulation and disease duration in our patients. Thus, it is possible that decreased ability to up-regulate Fas was a pre-existing condition in these patients. If this impairment in Fas up-regulation in T cells correlates with defective up-regulation of Fas in other tissues upon cellular stress, this impairment may confer predisposition to PD. Although the clinical data are in accordance with the results from our mouse study, it will be important to validate the clinical findings in a larger patient population.

Our studies demonstrate for the first time that a reduction in Fas expression or signaling dramatically increases neuronal susceptibility to neurodegeneration in vivo, strongly supporting a neuroprotective role for Fas, at least in some contexts. We propose that the lack of Fas in *lpr* mice and the decreased Fas signaling in *gld* mice is responsible for the increased neuronal death caused by the lack of a Fas-dependent neuroprotective signal. The striking level of dopaminergic neuron degeneration in the midbrain of MPTP-treated *lpr* mice, together with their profound behavioral phenotype, may provide a new model for PD research in which potential therapeutics and neuroprotective strategies can be tested. Moreover, individuals with an impaired ability to up-regulate Fas expression may be at increased risk of developing PD after exposure to environmental neurotoxins.

MATERIALS AND METHODS

Mice and MPTP treatment. We used 8–10-wk-old female B6 (Charles River Laboratories), *lpr*, *gld*, Fas exon 9 KO ("Fas null"), CBA, and *lpr.cg* mice (Jackson ImmunoResearch Laboratories). We administered five sub-

cutaneous injections of 25 mg/kg MPTP (Sigma-Aldrich) or saline, once per day for 5 consecutive d. Mice were used according to the protocols approved by McGill University Animal Care Committee.

Behavioral analysis. We performed automated activity monitoring (AM1053 system, Cambridge Neurotechnology Ltd.) over 5-min periods. We assessed Rotarod (IITC Life Science) performance as the average of three trials of 1 min each at 20 revolutions/min. Statistical analysis was by analysis of variance and Tukey test.

Brain preparation, immunohistochemistry, and stereology. We perfused mice, cut 50-μm coronal brain sections through the striatum and midbrain using a freezing sledge microtome (Leica), and performed immunohistochemistry with anti-TH antibody (1:2,000, Pel-Freez Biologicals) on every third serial section (22). A second series was processed using 0.1% cresyl violet as a Nissl stain. We obtained unbiased stereology estimates of midbrain dopaminergic neurons using StereoInvestigator software (MicroBrightField) as described previously (22).

Primary cultures. We prepared and treated P2–P5 midbrain cells from B6 mice with MPTP as described (23). FasL construct (400 ng/ml, Sigma-Aldrich) was administered where indicated 30 min before MPTP. After 7 d, TH^+ cells were stained with IgG-PE Zenon kit (Molecular Probes) bound to monoclonal mouse anti-TH antibody (Sigma-Aldrich).

Neuroprotection assay. We plated 2×10^4 Fas-positive SH-SY5Y neuroblastoma cells per well in phenol-red–free DMEM in 96-well plates. We pretreated the cells with FasL construct (100 ng/ml), added MPP^+ at the indicated concentrations 30 min later, then incubated the cells for 3 d at 37°C/5% CO_2. We assayed cell viability using WST-1 reagent (Roche Diagnostics) according to the manufacturer's instructions. As a control, the same procedure was performed on Fas-negative SH-SY5Y cells. IETD-fmk (BD Biosciences) was used where indicated and was tested by incubating 10^4 Jurkat cells/well at 37°C for 1 h with IETD-fmk to which FasL (100 ng/ml) was added for 2.5 h. WST-1 was used to assay viability.

HPLC. HPLC was performed as described (24) with modifications (see supplemental Materials and methods, available at http://www.jem.org/cgi/content/full/jem.20050163/DC1). Monoamines were detected with an ESA system with 5011 analytical cells, and a Higgins Analytical HAISIL 100 C18 column (5 μm, 150 × 4.6 mm). MPP^+ levels were measured using a Beckman 32 Karat System with a diode array detector (295 nm) on a Gemini C18 RP 150 × 4.6 mm analytical column (Phenomenex, Inc.).

Patients. We recruited patients who had idiopathic PD from five consecutive weekly Montreal Neurological Institute Movement Disorders Clinics. Diagnosis of idiopathic PD was established according to the following criteria: tremor, bradykinesia, and rigidity (two of three), asymmetric onset, response to dopaminergic medication (in patients taking medication), and absence of the following signs suggesting another diagnosis: early dementia, early falls or balance problems, severe autonomic dysfunction, and use of neuroleptic medications (25, 26). Patients with concurrent inflammatory diseases were excluded. Only men were included in this study, because we were unable to recruit enough women for statistical significance. Age-matched men with no history of PD or inflammatory disease were recruited as controls (average ages: patients with PD, 65.78 ± 6.94 yr; control subjects, 68.43 ± 15.41 yr). We obtained informed consent from all participants, and all experiments involving humans were approved by the Institutional Review Board of McGill University.

Flow cytometry. We stimulated mouse lymph node cells overnight with 2.5μg/ml Concanavalin A (Sigma-Aldrich) in RPMI 1640/10% FCS and then labeled the cells with PE-conjugated anti-mouse CD95 or PE-conjugated isotype-matched control antibodies (BD Biosciences). Human PBLs were prepared by ficoll density centrifugation from 10 ml blood. PBLs were divided into unstimulated cells and stimulated cells using Concanavalin A as described earlier. Cells were labeled with FITC-conjugated anti-human CD3 and PE-conjugated anti-human CD95 or PE-conjugated isotype-matched control antibodies (BD Biosciences). Fas up-regulation was defined as the change in mean PE fluorescence between the unstimulated and stimulated populations gated on live $CD3^+$ cells.

Online supplemental material. Videos 1–3 show mice 20 h after the second and third doses of MPTP. Mice and MPTP treatment were as described in Materials and methods. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050163/DC1.

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