nature immunology

Osteopontin-induced relapse and progression of autoimmune brain disease through enhanced survival of activated T cells

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Relapses and disease exacerbations are vexing features of multiple sclerosis. Osteopontin (Opn), which is expressed in multiple sclerosis lesions, is increased in patients' plasma during relapses. Here, in models of multiple sclerosis including relapsing, progressive and multifocal experimental autoimmune encephalomyelitis (EAE), Opn triggered recurrent relapses, promoted worsening paralysis and induced neurological deficits, including optic neuritis. Increased inflammation followed Opn administration, whereas its absence resulted in more cell death of brain-infiltrating lymphocytes. Opn promoted the survival of activated T cells by inhibiting the transcription factor Foxo3a, by activating the transcription factor NF-κB through induction of phosphorylation of the kinase IKKβ and by altering expression of the proapoptotic proteins Bim, Bak and Bax. Those mechanisms collectively suppressed the death of myelin-reactive T cells, linking Opn to the relapses and insidious progression characterizing multiple sclerosis.

One important characteristic of autoimmune disorders, which chronically afflict up to 8% of the population in the economically developed world, is the alternation of periods of remission and exacerbation, described as 'recurrent relapses'. These relapses contribute to the burden of chronic disability[1]. The mechanisms underlying the reactivation of disease, culminating in the exacerbation and progression of autoimmunity, remain elusive.

Osteopontin (Opn; also called 'early T cell–activation gene 1') is a multifunctional protein that has been linked to many physiological and pathological events, including bone remodeling, cancer and inflammation[2,3]. The much higher expression of Opn at the site of pathology in several autoimmune diseases, including multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis and inflammatory bowel disease, has focused attention on this molecule as a potentially critical factor in pathogenesis[4–10]. The involvement of Opn in autoimmunity has been emphasized in research on experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. Mice deficient in Opn (*Spp1*$^{-/-}$ mice; called 'Opn-knockout' mice here) have a milder clinical disease and their course of EAE does not show exacerbation or progression[4,11]. In concordance with those findings in animal models, patients with multiple sclerosis have increased Opn in the plasma before and during relapses of their disease[5,12].

The importance of Opn in autoimmune diseases can be partially explained by the proinflammatory functions of Opn, including the recruitment of leukocytes to sites of inflammation and polarization of the T helper type 1 cytokine response[13–15]. However, the finding that Opn-knockout mice with EAE have a degree of histological inflammation in the central nervous system similar to that of Opn-wild-type mice, despite having a milder clinical course[4], suggests that additional functions for Opn other than its proinflammatory effects may also be critical. Notably, involvement of Opn in cell survival has been reported for certain cell types, such as endothelial cells and pro–B cell lines[16,17], which raises the possibility that Opn affects T cell survival during immune responses to self and/or nonself antigens.

T cell death is pivotal in establishing self-tolerance and in preventing autoimmunity. The elimination of potentially self-reactive T cells is required both for central tolerance during thymic development and for peripheral tolerance[18]. Despite those two 'layers of protection' against the activation of autoaggression, undesirable activation of self-reactive T cells that escape those regulatory mechanisms poses a threat. Therefore, it is possible that the immune system incorporates further mechanisms to extinguish primary autoimmune attacks arising as a consequence of a break in tolerance to self. The apoptotic elimination of an already activated autoreactive T cell population after the initial autoimmune response might exist as one such mechanism for the deletion of deleterious T cells that escaped elimination in the thymus or in the periphery. In fact, T cell death occurs in the central nervous systems of mice with EAE at the time of spontaneous remission from paralytic disease[19–21].

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Received 17 July; accepted 24 October; published online 3 December 2006; doi:10.1038/ni1415

Here we explore a previously unknown mechanism whereby Opn exacerbates EAE by promoting the survival of activated autoreactive T cells. Using three different multiple sclerosis models, we found that Opn mediated relapses and induced a shift to what some consider the secondary or progressive stage of this autoimmune disease of the brain[22]. Furthermore, our results indicate that Opn promotes the survival of activated T cells through regulation of the transcription factors Foxo3a and NF-κB and through the expression of proapoptotic proteins.

RESULTS

Opn induces relapses and progression of EAE

Increased Opn expression has been reported in many autoimmune diseases, including multiple sclerosis, systemic lupus erythematosus and rheumatoid arthritis[4–9,12]. Thus, we attempted to elucidate the function of Opn using an autoimmune disease model. The various EAE models of multiple sclerosis are of particular interest because it has been shown that Opn-knockout mice with EAE have spontaneous remissions after the initial attack of paralysis in EAE[4].

We induced acute EAE in Opn-knockout and Opn-wild-type mice using myelin oligodendrocyte glycoprotein (MOG) peptide (amino acids 35–55). To test whether Opn can actively exacerbate clinical paralysis, we administered recombinant Opn (rOPN) to Opn-knockout mice with EAE. We began injecting mice during the first spontaneous recovery of the disease in each Opn-knockout mouse, defined by a decrease in clinical score for 2–4 consecutive days after the first peak of the disease. To mimic the condition of patients with multiple sclerosis who have highly increased plasma concentrations of Opn before a clinical relapse of their disease[5,12], we administered rOPN intravenously to the mice daily. Notably, the ongoing remission of the disease was reversed and the extent of clinical paralysis worsened over time after rOPN administration. The recrudescence of EAE induced after the administration of rOpn was followed by progression to severe disease, ultimately leading to death. All rOpn-treated mice (seven of seven) died from EAE within 35 d of the administration (**Fig. 1a** and **Supplementary Table 1** online). These results indicated that Opn inhibits spontaneous recovery and directly mediates clinical exacerbation and progression.

We next assessed the effect of the administration of rOpn in a relapsing-remitting model of EAE, using SJL/J mice immunized with a peptide of proteolipid protein (amino acids 139–151). Intravenously administered rOPN injected during the first recovery as described above induced a rapid relapse with an increased degree of paralysis and corresponding increase in clinical score from that noted during remission. Overall, there was a worsening of the clinical course with a gradual increase in the average minimum and maximum scores during successive cycles of relapse and remission in this model (**Fig. 1b**). The fraction of mice with complete recovery, in which the clinical score returned to zero, was decreased by rOpn treatment in every time period. The inhibition of spontaneous recovery by Opn in relapsing EAE was evident as early as 1 d after the administration of rOpn and was observable for 15 d. In that period, fewer mice in the rOpn-treated group (33%) than in the PBS-treated group (55.6%) showed complete recovery. During a later time period, from day 31 to day 45 of treatment, eight of nine (88.9%) of mice in the PBS-treated group had still recovered completely, whereas in the rOpn-treated group, only one of nine mice had recovered. Those findings suggested that Opn induced a gradual shift from the relapsing-remitting stage to the chronic progressive stage (**Table 1**).

We also tested whether the initiation of EAE by repeated activation of autoreactive T cells was augmented in the mice that received rOPN. For that, we gave transgenic mice expressing MOG-specific T cell receptors (TCRs)[23] primary and secondary immunization with the

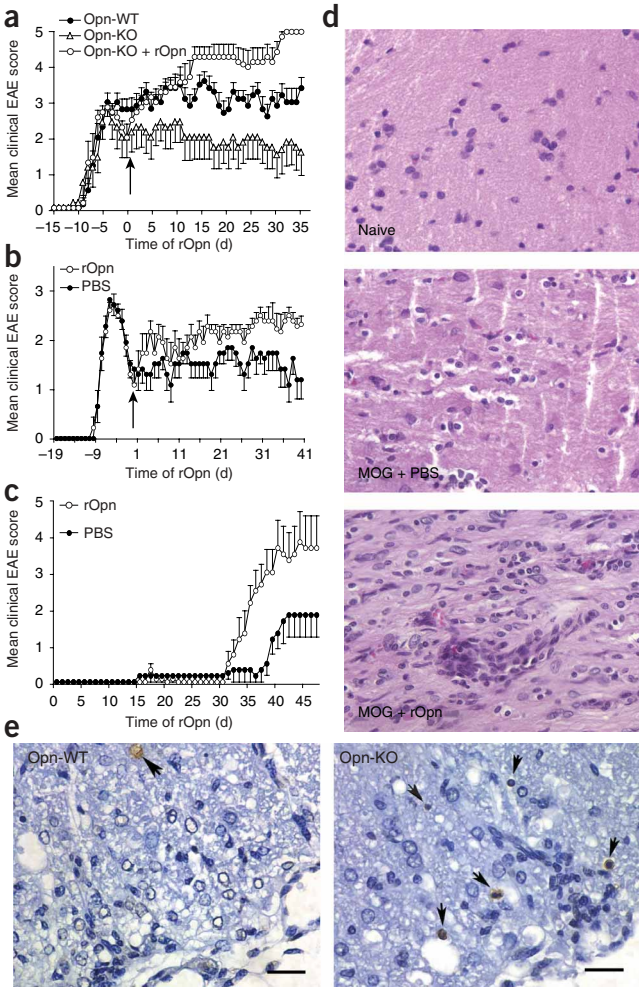

Figure 1 Opn induces worsening autoimmune relapses and severe progression of autoimmune demyelinating disease. (**a**) Clinical scores of EAE induced by immunization of Opn-wild-type mice (Opn-WT; $n = 10$) and Opn-knockout mice[4] (Opn-KO; $n = 14$) with MOG peptide; half of the Opn-knockout mice ($n = 7$) were given rOPN daily for 32 d after the initial peak of the clinical disease, initiated during the first remission of each mouse. (**b**) Clinical scores of female SJL/J mice immunized with the peptide of proteolipid protein and then treated with PBS ($n = 9$ mice) or with rOPN ($n = 9$ mice) as described in **a**. Upward arrows (**a**,**b**), first day of rOpn treatment. (**c**) Clinical scores of MOG-specific TCR–transgenic mice[23] given primary immunization with MOG peptide without pertussis toxin and then treated with PBS ($n = 6$ mice) or with rOPN ($n = 6$ mice) daily beginning on the day of primary immunization; mice were immunized with MOG peptide plus pertussis toxin 25 d after primary immunization. (**d**) Hematoxylin-and-eosin staining of optic nerve tissue sections isolated from MOG-specific TCR–transgenic mice with optic neuritis and EAE. Naive, no MOG immunization. Original magnification, ×200. (**e**) TUNEL staining of brain and spinal cord tissue from Opn-knockout and Opn-wild-type mice immunized with MOG peptide to induce EAE, obtained on day 17 after immunization. Arrows indicate TUNEL-positive (brown) nuclei of infiltrating lymphocytes stained with 3,3′-diaminobenzidine. Scale bars, 25 μm. Data represent mean clinical score (+ s.e.m.) of three experiments (**a**–**c**) and images are of representative tissues from three (**d**) or two (**e**) experiments.

Table 1 EAE in SJL/J mice treated with rOPN

	Complete recovery (%)			Mean minimum score				Mean maximum score		
	Time after treatment (d)			Time after treatment (d)				Time after treatment (d)		
	1–15	16–30	31–45	0	1–15	16–30	31–45	1–15	16–30	31–45
rOPN	33.3% (3 of 9)	11.1%† (1 of 9)	11.1%‡ (1 of 9)	1.1 ± 0.3	1.4 ± 0.4	1.7 ± 0.3*	1.9 ± 0.3***	2.2 ± 0.2	2.7 ± 0.2**	3.0 ± 0.2**
PBS	55.6% (5 of 9)	66.7%† (6 of 9)	88.9%‡ (8 of 9)	1.4 ± 0.3	0.9 ± 0.4	0.7 ± 0.3*	0.2 ± 0.2***	2.0 ± 0.0	2.1 ± 0.1**	2.4 ± 0.2**

Mice were treated daily for various periods of time with PBS or with rOpn at a dose of 5 μg per mouse. 'Complete recovery' is defined as a decrease of the clinical score to zero; in parentheses are number of mice with complete recovery/total number of mice. Minimum and maximum scores are mean ± s.e.m.
†, $P = 0.025$, and ‡, $P = 0.0017$ (Fisher exact-probability test); *, $P < 0.1$, **, $P < 0.05$, and ***, $P < 0.0005$ (ANOVA).

cognate MOG peptide (amino acids 35–55) and, in concert, intravenously administered rOPN daily from the initial day of primary immunization. Those 'MOG-specific TCR–transgenic mice' (2D2 mice) treated with rOPN developed more severe and fulminant EAE than did the PBS-treated control mice. That more-severe clinical score was associated with higher mortality (66.7% versus 0%) and accelerated onset (day 9 versus day 14; **Fig. 1c** and **Table 2**) and with more-severe central nervous system inflammation (**Fig. 1d** and **Supplementary Fig. 1** online).

Furthermore, in this experiment, all mice (six of six) in the rOpn-treated group developed tearing or atrophy of the eye and eyelid swelling, which are clinical signs of optic neuritis (**Table 2** and **Supplementary Fig. 1**), whereas only one of six mice in the control group developed those signs, with later onset. Optic neuritis is one of the hallmark presentations of multiple sclerosis and other related demyelinating diseases, including neuromyelitis optica. Given a published report noting the development of spontaneous autoimmune optic neuritis in a proportion (35%) of older MOG-specific TCR–transgenic mice at the approximate mean age of 6 months (ref. 23), the appearance of optic neuritis with higher incidence (100%) in those much younger mice that were 5 weeks old at the time of initial immunization was probably due to the rOpn administered over the course of primary and secondary immunizations with MOG peptide. The enhanced severity and accelerated onset of EAE with increased incidence of clinical optic neuritis accompanying rOpn administration strongly suggested that Opn augments autoreactive T cell responses after reactivation with MOG peptide. These experiments collectively indicated that mice with increased Opn concentrations are much more susceptible to the development of autoimmune central nervous system diseases when they are exposed to repeated activation of autoreactive T cells.

To address the potential contribution of endotoxin present in the purified rOPN, we also administered the endotoxin lipopolysaccharide to mice with EAE (**Supplementary Fig. 2** online). The residual endotoxin concentration in the rOPN we used was less than 0.003 endotoxin units per microgram of purified protein, as analyzed by a standard limulus amoebocyte lysate assay. The clinical scores of SJL/J and MOG-specific TCR–transgenic 2D2 mice with EAE that received 0.5 ng lipopolysaccharide were not different from those of the PBS-treated group. Thus, the residual lipopolysaccharide in the purified protein did not contribute the effect of Opn on EAE described above.

Given that apoptosis of infiltrating T cells in lesions has been noted during clinical recovery in autoimmune diseases of the nervous system, including animal models of multiple sclerosis and Guillain-Barré syndrome[20], we postulated that the milder clinical signs of Opn-knockout mice with EAE might be related to increased cell death of pathogenic lymphocytes infiltrating the central nervous system. We therefore assessed cell death in the affected tissues of EAE in Opn-wild-type and Opn-knockout mice. We did TUNEL (terminal deoxinucleotidyltransferase-mediated dUTP-biotin nick end-labeling) assays of central nervous system tissue sections obtained during spontaneous clinical recovery after the peak of disease. We found more TUNEL-positive cells in the parenchymal inflammatory foci of Opn-knockout mice with EAE than in those of Opn-wild-type mice with EAE (**Fig. 1e**). Thus, the absence of Opn resulted in enhanced cell death of leukocytes *in situ*.

Those results indicated that Opn might modulate the apoptotic elimination of infiltrating lymphocytes occurring after an initial inflammatory autoimmune response in the central nervous system. Substantial expression of Opn has been found in active multiple sclerosis plaques and in the inflammatory lesions of relapsing-remitting EAE[4]. Thus, it is plausible that the elimination of autoreactive T cells is inhibited by Opn in central nervous system inflammatory lesions *in vivo*.

Enhanced survival of activated T cells by Opn

Next we assessed the effect of Opn on activated T cell death *in vitro* by adding rOPN to cultures of stimulated T cells. To monitor cell death, we measured DNA fragmentation by the TUNEL assay. The rOpn reduced the percentage of TUNEL-positive apoptotic T cells in both

Table 2 EAE and autoimmune optic neuritis induced by Opn in MOG-specific TCR–transgenic mice

	EAE				Optic neuritis	
	Incidence	Mortality	Mean time of onset (d)	Mean maximum score	Incidence	Mean time of onset (d)
rOPN	83% (5 of 6)	67% (4 of 6)	9.0 ± 0.9*	4.0 ± 0.8	100% (6 of 6)	15.7 ± 2.1
PBS	67% (4 of 6)	0% (0 of 6)	14.3 ± 0.3*	1.8 ± 0.6	17% (1 of 6)	17.0 ± 0.0

MOG-specific TCR–transgenic mice were immunized with MOG peptide and were injected with rOpn or PBS as described in **Figure 1c**. Onset of disease (mean ± s.e.m.) is defined as days from the secondary MOG peptide immunization. Clinical scores are mean maximum (± s.e.m.) for mice showing clinical disease. Numbers in parentheses are number of mice that developed EAE or optic neuritis or died/total number of mice.
*, $P = 0.0017$ (ANOVA).

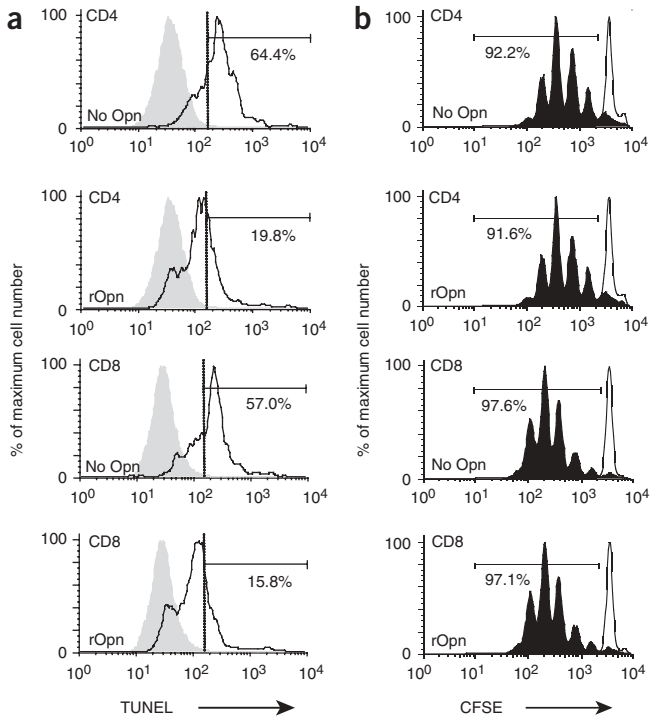

Figure 2 Opn inhibits cell death but does not affect cell division. (**a**) TUNEL assay of the death of Opn-wild-type lymph node T cells stimulated with concanavalin A and cultured for 24 h in the presence or absence of rOPN (2 μg/ml). Stimulated T cells (solid lines) and unstimulated control T cells (filled histograms) were analyzed by flow cytometry. Numbers below bracketed lines indicate percent TUNEL-positive cells. (**b**) Flow cytometry of the CFSE profiles of cultured $CD4^+$ and $CD8^+$ T cells. CFSE-labeled splenocytes from Opn-knockout mice were stimulated for 72 h as described in **a** in culture with or without rOPN (2 μg/ml); the CFSE fluorescence of unstimulated cells (solid lines) and stimulated cells (filled histograms) was analyzed after gating on $CD4^+CFSE^+$ or $CD8^+CFSE^+$ cells. Numbers below bracketed lines indicate percent dividing cells. Data are representative of three independent experiments.

$CD4^+$ and $CD8^+$ T cell subsets after activation (**Fig. 2a**). Also, rOpn added in culture increased the antigen-specific [^{3}H]thymidine incorporation of $CD4^+$ T cells specific for the Ac1-11 pathogenic epitope of myelin basic protein (**Supplementary Fig. 3** online). The increase in [^{3}H]thymidine incorporation could have been due to decreased cell death and/or increased cell division. To address those possibilities, we analyzed the cell division of activated T cells using splenic T cells labeled with CFSE (carboxyfluorescein diacetate succinimidyl diester) and cultured with rOpn. To minimize the effect of endogenously secreted Opn, we used cells from Opn-knockout mice for this analysis. For both $CD4^+$ and $CD8^+$ T cells, the cell division profile and the percentage of cycling cells were not altered substantially by additional rOpn (**Fig. 2b**), indicating that Opn does not enhance cell division. We confirmed that finding by cell cycle analysis using 5-bromodeoxyuridine and 7-amino-actinomycin D (7-AAD), in which we obtained similar results (data not shown). Thus, these results demonstrated that Opn inhibits T cell death without influencing cell division.

To further investigate the molecular mechanism underlying Opn-induced survival of activated T cells, we examined whether Opn affects the activity of proteins involved in cell survival in activated T cell signal transduction. Opn induces the activity of phosphatidylinositol 3-OH kinase and phosphorylation of the serine-threonine kinase Akt (also called 'protein kinase B') in breast cancer cell lines and in a pro–B cell line in relation to cell migration[24,25]. We examined the activity of the kinase PDK1, which activates Akt, and of PTEN, a negative regulator of phosphatidylinositol 3-OH kinase–Akt signaling, as well as of Akt, by assessing phosphorylation of those molecules in the presence of exogenously added rOpn in cultures of activated T cells. Phosphorylation of PDK1, PTEN and Akt was not affected by the addition of rOpn to primary T cells activated by CD3 and CD28 stimulation (**Fig. 3a**). Although it has been reported that Opn induces Akt phosphorylation in some tumor cell lines, Akt phosphorylation was not increased substantially in activated primary T cells by Opn, as assessed in our experimental conditions.

Next we further investigated whether Opn affects other 'downstream' effectors involved in the phosphatidylinositol 3-OH kinase and Akt pathway. The forkhead family transcription factor Foxo3a induces the transcription of target genes that encode proteins involved in DNA damage repair and apoptosis. As Foxo3a is one of the downstream substrates of Akt and the transcriptional activity of Foxo3a is regulated by phosphorylation, which serves as a nuclear exclusion signal[26,27], we analyzed the phosphorylation status of Foxo3a in the presence of Opn. An inactive, phosphorylated form of Foxo3a was increased in the presence of rOPN added in the culture. We noted that phosphorylation of Foxo3a by rOpn in activated T cells after stimulation with CD3 plus CD28 and after stimulation with concanavalin A (**Fig. 3b**). Foxo3a phosphorylation was more dose sensitive to rOpn after concanavalin A stimulation. These results indicated that Opn inhibits the transcriptional activity of Foxo3a, which is a transcription factor inducing the expression of genes involved in proapoptosis.

We also investigated the effect of Opn on the activation of NF-κB, which is important in many pathways, including cell survival and the induction of T helper type 1 cytokines. The activation of NF-κB, as assessed by degradation of the NF-κB inhibitor IκBα, was induced by Opn (**Fig. 3c**). We also directly tested the activation of NF-κB by Opn by assessing DNA binding of the subunits p65 and p50 (**Fig. 3d**). Opn enhanced and antibody to Opn (anti-Opn) suppressed the activation of NF-κB in activated T cells.

We next examined whether Opn also affects IKKβ, the kinase 'upstream' of IκBα. Active IKKβ phosphorylates IκBα and induces the degradation of phosphorylated IκBα. Immunoblot analysis showed that IKKβ phosphorylation was increased in the presence of high concentrations of Opn within 24 h of T cell activation (**Fig. 3e**). Our results indicated that active NF-κB formation triggered by IκB degradation was induced by Opn through IKKβ phosphorylation. Furthermore, phosphorylated, active IKKβ is known to phosphorylate Foxo3a as well[28]. Thus, it is likely that both increased phosphorylation of Foxo3a and degradation of IκBα were mediated by IKKβ in Opn signaling. These results collectively indicated that Opn inhibits the transcriptional activity of Foxo3a by increasing phosphorylation, while at the same time it induces the activation of NF-κB in activated T cells. Notably, both downstream events of Opn signaling can be mediated by active IKKβ and contribute to the prosurvival function of Opn in T cells, as the target genes of Foxo3a include those encoding proapoptotic proteins such as Bim[29] and the target genes of NF-κB include many encoding prosurvival proteins[30].

It has been reported that a group of proteins designated 'BH3-only Bcl-2 family proteins', including Bim (Bcl-2-interacting molecule), and another group of proteins designated 'multi-BH domain proteins', including Bak and Bax, are critical in cell death, driving mitochondrial dysfunction[31]. Bim activates Bak and Bax, and that activation is

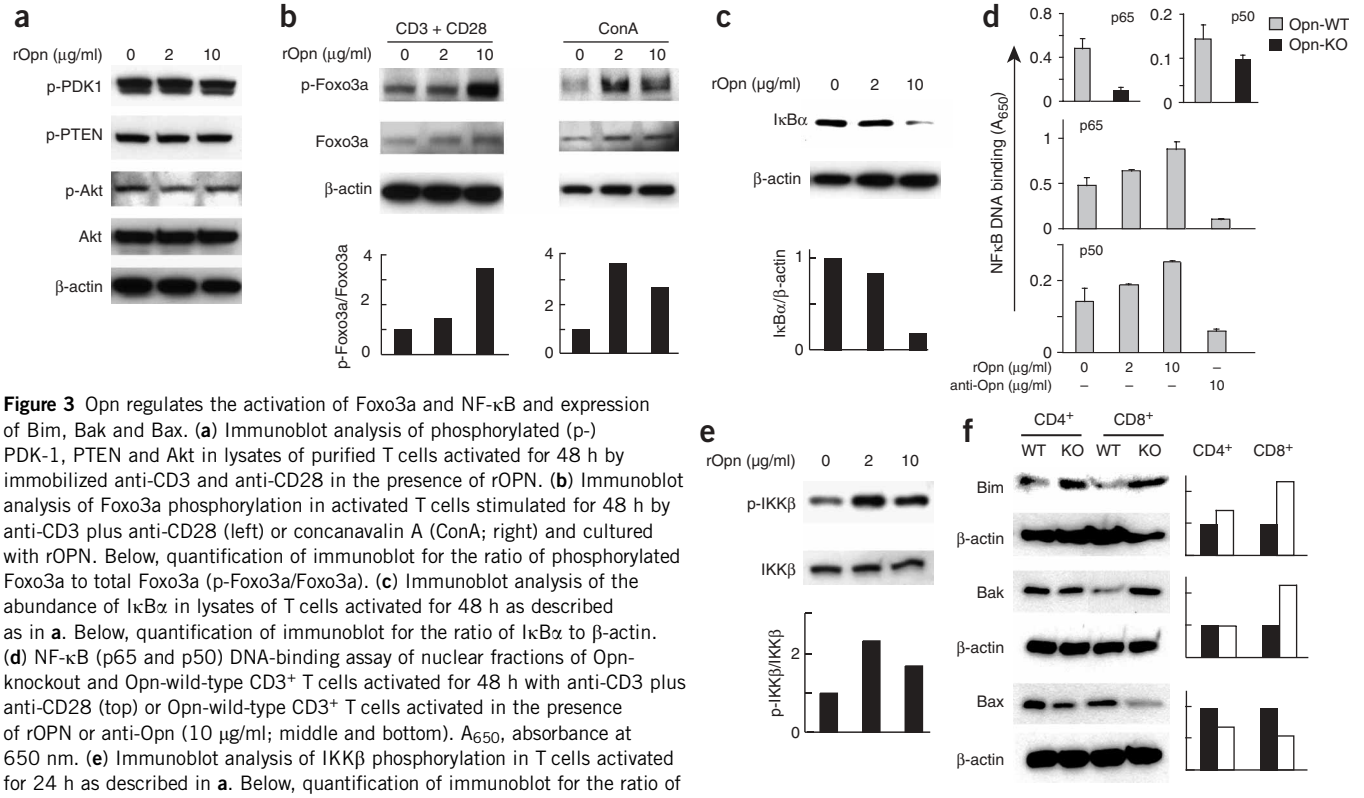

Figure 3 Opn regulates the activation of Foxo3a and NF-κB and expression of Bim, Bak and Bax. (**a**) Immunoblot analysis of phosphorylated (p-) PDK-1, PTEN and Akt in lysates of purified T cells activated for 48 h by immobilized anti-CD3 and anti-CD28 in the presence of rOPN. (**b**) Immunoblot analysis of Foxo3a phosphorylation in activated T cells stimulated for 48 h by anti-CD3 plus anti-CD28 (left) or concanavalin A (ConA; right) and cultured with rOPN. Below, quantification of immunoblot for the ratio of phosphorylated Foxo3a to total Foxo3a (p-Foxo3a/Foxo3a). (**c**) Immunoblot analysis of the abundance of IκBα in lysates of T cells activated for 48 h as described as in **a**. Below, quantification of immunoblot for the ratio of IκBα to β-actin. (**d**) NF-κB (p65 and p50) DNA-binding assay of nuclear fractions of Opn-knockout and Opn-wild-type $CD3^+$ T cells activated for 48 h with anti-CD3 plus anti-CD28 (top) or Opn-wild-type $CD3^+$ T cells activated in the presence of rOPN or anti-Opn (10 μg/ml; middle and bottom). A_{650}, absorbance at 650 nm. (**e**) Immunoblot analysis of IKKβ phosphorylation in T cells activated for 24 h as described in **a**. Below, quantification of immunoblot for the ratio of phosphorylated IKKβ to total IKKβ. (**f**) Immunoblot analysis of the expression of Bim, Bak and Bax in purified naive Opn-wild-type (WT) and Opn-knockout (KO) $CD4^+$ and $CD8^+$ T cells. Right, quantification of immunoblot at left for expression relative to that of β-actin: filled bars, Opn-wild-type; open bars, Opn-knockout. Results (**a**–**f**) are representative of three independent experiments.

hindered by Bcl-2 and Bcl-x_L, the prosurvival Bcl-2 family members, which directly bind to Bim[32]. Therefore, in this pathway, cell death is probably regulated by the quantitative balance between proapoptotic and antiapoptotic Bcl-2 family proteins[33].

Bim is encoded by one of the target genes of Foxo3a[29], which we found here to be regulated by Opn. We next assessed the expression of Bcl-2 family proteins in Opn-knockout T cells. Notably, resting Opn-knockout $CD4^+$ and $CD8^+$ T cells had higher expression of Bim than did their Opn-wild-type counterparts. Moreover, the expression of both Bak and Bax was also altered in Opn-knockout T cells (**Fig. 3f**).

We further examined the expression profiles of these proteins induced by T cell activation. The expression of Bim and Bak was upregulated after T cell activation over a 72-hour period, whereas Bax expression was changed only slightly over time. The expression kinetics derived from the immunoblot analysis showed that the expression profiles of those proteins after T cell activation were very different in Opn-knockout versus Opn-wild-type cells (**Supplementary Fig. 4** online). In Opn-knockout mice, the increased Bim expression at the resting stage diminished over the 48-hour period of T cell activation, but Bim expression was induced again after that time. We noted a similar trend for Bak expression, except that we did not find reinduction during the later time period. Bax expression showed a substantial change in Opn-knockout cells as well, with lower expression in the resting stage and greatly induced expression after 48 h. Collectively, these results suggested that Opn modulates expression of the proapoptotic proteins Bim, Bak and Bax. We also assessed expression of the antiapoptotic proteins Bcl-2 and Bcl-x_L; however, we were unable to detect distinct changes for Opn-knockout versus Opn-wild-type cells (data not shown).

We next used antibodies to Fas (CD95) *in vitro* to assess Fas-mediated Opn-knockout T cell death. Flow cytometry showed that the apoptosis of Opn-knockout T cells induced by Fas crosslinking was similar to that of Opn-wild-type T cells for both the $CD4^+$ and $CD8^+$ subpopulations (**Supplementary Fig. 5** online). In agreement with that result, the expression of both Fas and Fas ligand was similar on the surfaces of Opn-knockout and Opn-wild-type T cells (data not shown). That result indicated that Opn does not affect the Fas-mediated death of activated T cells.

Mode of cell death inhibited by Opn

Massive caspase activation is frequently found in apoptosis, often associated with mitochondrial instability. We tested whether the enhancement of the survival of T cells by Opn was associated with the inhibition of caspase activation. We explored that issue using the caspase inhibitor z-VAD-fmk in the TUNEL assay to assess the death of activated T cells. Analysis of activated T cells cultured with z-VAD-fmk demonstrated that inhibition of caspases was not sufficient to 'rescue' activated Opn-knockout $CD4^+$ T cells from their increased death (**Fig. 4a**). In a kinetic study of T cell survival after activation, Opn deficiency, which was associated with profound death of $CD4^+$ T cells, began early, whereas Opn-wild-type $CD4^+$ T cells seem more resistant to that early death. In addition, caspase dependency was more profound for the death of $CD4^+$ T cells than that of $CD8^+$ T cells from Opn-wild-type mice (**Fig. 4b**). Collectively, these results suggested that Opn-knockout T cells were predisposed to death, more prominently for $CD4^+$ T cells, and that the cell death occurred in a caspase-independent way, modulated via Opn.

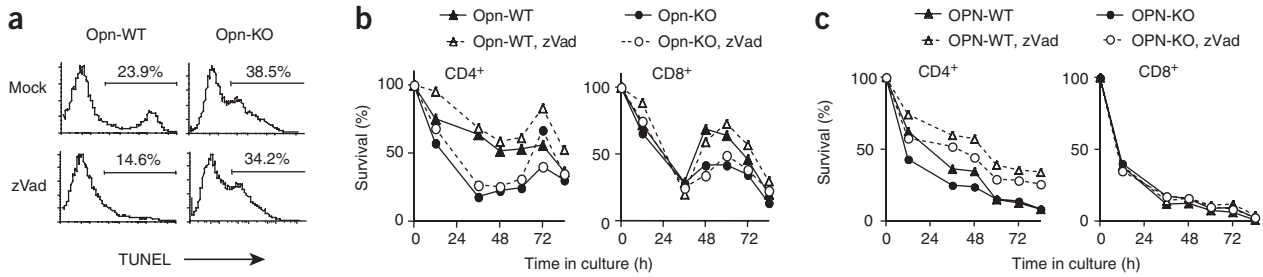

Figure 4 Mode of cell death inhibited by Opn in T cell. (**a**) TUNEL assay of the death of Opn-wild-type or Opn-knockout lymph node T cells stimulated with concanavalin A in the presence (zVad) or absence (Mock) of z-VAD-fmk, assayed 24 h after activation; TUNEL-positive $CD4^+$ T cells were assessed by flow cytometry as described in **Figure 2a**. Numbers above bracketed lines indicate TUNEL-positive cells. (**b**,**c**) Flow cytometry to assess the survival of Opn-wild-type or Opn-knockout $CD4^+$ and $CD8^+$ lymph node T cells either activated as described in **a** (**b**) or resting (naive; **c**); cells were cultured in the presence or absence of z-VAD-fmk and were stained with anti-CD4 or anti-CD8 every 12 h, and the percent live cells was determined by propidium iodide staining at various times (horizontal axis) after activation. Data (**a**–**c**) are representative of two independent experiments.

We also found that Opn deficiency differentially influenced the viability of resting $CD4^+$ and $CD8^+$ T cells (**Fig. 4c**). Resting Opn-knockout $CD4^+$ T cells were less viable than were Opn-wild-type $CD4^+$ T cells without stimulation in culture, whereas the death of resting $CD8^+$ T cells was not affected by Opn deficiency. Notably, the survival of resting $CD4^+$ T cells was increased by caspase inhibition for both Opn-knockout and Opn-wild-type cells, indicating that the activity of caspase is important in that mode of spontaneous death of resting T cells. These observations suggested that Opn also promotes the survival of naive $CD4^+$ T cell but does not affect naive $CD8^+$ T cells.

Release of mitochondrial proteins is a result of mitochondrial dysfunction in different modes of cell death. Apoptosis-inducing factor (AIF) is a flavoprotein released from mitochondria in response to various stimuli inducing cell death[34]. AIF has been reported to have a dual function in cell survival. Notably, although several publications have indicated that AIF is important in apoptosis, even in the apoptosis of oligodendrocytes[35], some investigators have suggested that AIF may not have a critical and direct function in apoptosis but may merely be a 'surrogate marker' for biochemical changes associated with this process[36] (D. Green, personal communication). Studies supporting the idea of an important physiological function for AIF in apoptosis have demonstrated that when it is localized in mitochondria, the oxidoreductase activity of AIF protects cells from oxidative stresses, promoting cell survival; however, nuclear translocation of AIF occurs after cell death and is associated with chromatin condensation and massive DNA fragmentation[35,37].

We examined the effect of Opn on the nuclear translocation of AIF in activated T cell death using Opn-knockout splenocytes with rOPN added in culture. Subcellular fractionation and immunoblot analysis showed that the nuclear localization of AIF was much higher in the early phase of T cell activation in Opn-knockout cells (**Supplementary Fig. 6** online). AIF expression in the nucleus was induced after activation of Opn-wild-type splenocytes. In contrast, in Opn-knockout mice, the nuclear AIF abundance constitutively increased in the resting state and was maintained until 24 h after activation. That increase occurred in the mitochondria as well, demonstrating that the regulation of AIF expression was impaired in Opn-knockout splenocytes. Also, the temporal decrease in nuclear AIF was rapidly restored in Opn-knockout cells within 24 h. That observation suggested that Opn deficiency promotes the expression and nuclear localization of AIF. We also directly confirmed the salient inhibitory effect of Opn on the nuclear translocation of AIF using rOPN in cultures of activated T cells (**Supplementary Fig. 6**). Whether more nuclear localization of AIF sensitizes Opn-knockout T cells to programmed cell death or whether the action of Opn in regulating the nuclear versus mitochondrial location of AIF merely reflects the trafficking and subcellular localization of AIF as a surrogate marker of apoptosis, it is apparent that Opn influences the localization of this protein.

Thus, our cellular and biochemical studies have collectively demonstrated that Opn is critical in T cell survival by 'rescuing' those cells from programmed cell death induced by T cell activation as follows (**Supplementary Fig. 7** online): First, Opn prevents T cell death by regulating the activity of Foxo3a, which induces genes encoding proapoptotic proteins. Second, Opn promotes cell survival by inducing NF-κB activation. Third, Opn affects the expression of the proapoptotic Bcl-2 proteins Bim, Bak and Bax.

'Postimmune extermination' of activated T cells and Opn

Finally, we confirmed the prosurvival activity of Opn *in vivo* using adoptive transfer experiments. We labeled T cells from the Opn-knockout and Opn-wild-type mice with CFSE and transferred them into lymphopenic (recombination-activating gene 1–deficient ($Rag1^{-/-}$)) recipient mice and then analyzed the survival of the transferred T cells by flow cytometry on day 8 after transfer. Consistent with our other results, the survival rate of Opn-knockout T cells was lower than that of Opn-wild-type T cells in the $Rag1^{-/-}$ host mice (**Fig. 5a**). Notably, that lower survival rate was more prominent when we transferred activated rather than naive T cells, suggesting that the survival was sensitive to Opn to a greater degree for activated T cells *in vivo* (**Fig. 5**). We also addressed the importance of the Opn produced from different sources in this transfer experiment. The lower survival of Opn-knockout T cells in the $Rag1^{-/-}$ host mice, in which other sources of Opn such as macrophages can produce Opn normally, indicated that Opn secreted from T cells is critical in T cell survival and may function in an autocrine or paracrine way.

Here we have shown that mice in three different models of EAE, when treated with Opn, have more-severe clinical courses associated with worsening of disease and more-severe relapses culminating in death. The overall profile of disease progression of these mice is reminiscent of secondary progressive multiple sclerosis, which is characterized by steady disease progression. Most patients with relapsing-remitting multiple sclerosis develop the secondary progressive phase through recurring relapses[22]. In secondary progressive multiple sclerosis, although isolated attacks are often accompanied by spontaneous remissions, the clinical recoveries are incomplete, and gradual exacerbation of the symptoms appears between attacks[38]. The underlying mechanism for the progression of the disease through recurrent relapses is one of the key unanswered issues in research on

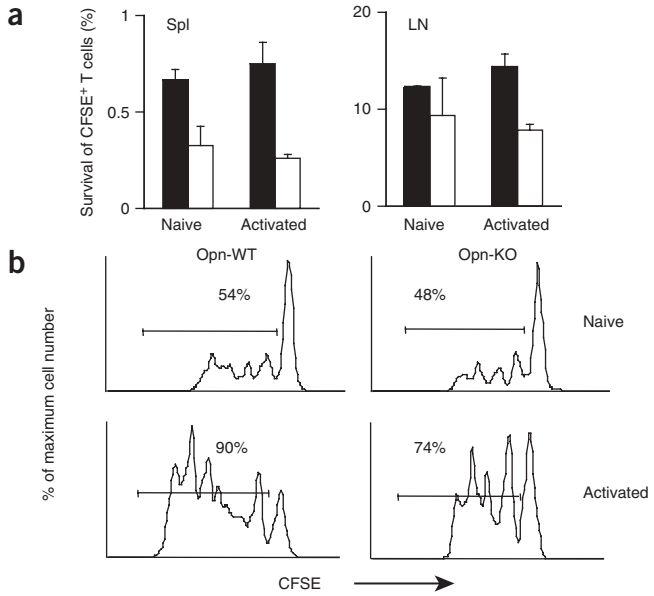

Figure 5 Opn promotes the survival of adoptively transferred T cells *in vivo*. $CD3^+$ T cells were purified from the lymph nodes of Opn-knockout or Opn-wild-type donor mice, then naive or activated T cells (3×10^6 cells) labeled with CFSE were adoptively transferred intravenously into syngeneic *Rag1*$^{-/-}$ recipient mice; at day 8 after transfer, transferred $CFSE^+$ T cells from the spleen and lymph nodes from the recipient mice were analyzed by flow cytometry to assess survival and proliferation. (**a**) Percent survival (mean ± s.e.m.) of $CFSE^+$ T cells from Opn-wild-type donors (filled bars) or Opn-knockout donors (open bars) in spleen (Spl) or lymph nodes (LN) of recipient mice. $n = 3$ recipient mice. (**b**) CFSE fluorescence of naive or activated T cells transferred from Opn-wild-type or Opn-knockout donors and isolated from lymph nodes of recipient mice. Numbers above bracketed lines indicate percent dividing cells. Data are representative of two experiments.

autoimmune diseases. Our results here demonstrating the unexpected potency of Opn in autoimmune disease along with Opn-induced T cell survival suggest that inflammatory autoimmune responses may be 'quenched' by the programmed death of autoreactive cells during spontaneous remission. That process is inhibited by Opn secreted from activated T cells, especially in patients with multiple sclerosis who have much more Opn in brain lesions and in plasma before and during relapse. Given that Opn mediates the clinical relapse in an EAE model, we propose a possible model for autoimmune relapse (**Supplementary Fig. 8** online). The apoptotic extermination of autoreactive T cells activated as a result of a break in tolerance in certain conditions may occur as an alternative to or as an 'extra layer' of the protection from severe autoimmunity. The effective postimmune extermination of autoimmune T cells may contribute to minimizing the risks of secondary autoimmune responses. In our model, the prosurvival function of Opn may elicit the incomplete postimmune extermination of autoreactive T cells, leading to relapses in autoimmune diseases. The process of postimmune extermination of self-reactive T cells could provide an important control measure for removing pathogenic T cells to limit tissue damage. Therefore, it seems that T cell death may be key not only in preventing autoimmunity through self-tolerance but also in extinguishing autoimmune activation after disease is initiated. One of the notable features of autoimmune diseases is their chronicity, often lasting the entire adult life of the patient.

DISCUSSION

Mechanisms other than those described above have been shown to be associated with relapses of disease. Microbial 'superantigens' are reported to trigger relapses of EAE[39]. The diversification of immune responses through epitope spreading has also been described in association with relapses of EAE and has been linked to the pathogenesis of relapses[40]. Tolerization to the priming antigen has been suggested by some studies[41] to be more critical in controlling relapses, although others have shown that amelioration of subsequent progression can be accomplished by tolerizing to an epitope on a secondary target of the immune response[40,41]. In any case, Opn can induce exacerbation in animal models of multiple sclerosis and it has been associated with relapses in the human disease.

Furthermore, on the basis of our model, it is conceivable that autoreactive memory T cells differentiated as a consequence of the failure of a complete postimmune extermination process may be involved in the reactivation of autoimmunity. Therefore, autoimmune relapse may be the result of reactivation of an autoreactive memory T cell. Notably, the autoreactive memory T cell response is increased exclusively in the relapse stages of the relapsing-remitting EAE model[42].

Our findings have suggested that high Opn expression actively exacerbates the relapse and progression of autoimmune disease. We have also identified Opn as a critical cytokine that 'saves' activated T cells from death. Although some other cytokines, such as tumor necrosis factor, interleukin 1 (IL-1) and transforming growth factor-β, have been also linked to the regulation of activated T cell death[33], their functions *in vivo* and the underlying mechanisms are still unclear. Moreover, as tumor necrosis factor and transforming growth factor-β paradoxically have opposing effects on T cell survival[43,44], it is difficult to clarify their functions as survival factors. Notably, as tumor necrosis factor, IL-1 and transforming growth factor-β all induce expression of the gene encoding Opn[45], their antiapoptotic effects might be mediated through Opn.

We have found that Foxo3a and NF-κB were reciprocally regulated by Opn. After an Opn signal, highly phosphorylated Foxo3a was excluded from the nucleus and was inactivated. In contrast, Opn increased IκBα degradation, triggering transcriptional activation and nuclear translocation of NF-κB. The finding that Opn activated IKKβ provides a mechanistic basis for explaining the control of both transcription factors in response to Opn. That dynamic regulation of Foxo3a and NF-κB controls the balance of the death and survival of activated T cells.

In our results, Opn altered expression of the proapoptotic proteins Bim, Bak and Bax. The expression kinetics of those proteins were also different in Opn-knockout versus Opn-wild-type mice. Their expression kinetics were differentially regulated in Opn-knockout mice in our experimental conditions. Consistent with our results, the sequential and differential modulation of Bak and Bax has been reported before in various different conditions of apoptosis, such as drug-induced apoptosis of cancer cells, IL-12 treatment of T cells and the apoptosis of leukemic cells induced by the proapoptotic molecule TRAIL[46,47]. The data showing that the cytosolic protein Bax was activated and translocated to mitochondria later than the activation of Bim and Bak, which are located in mitochondria in T cells, supported the idea that Bak may be more critical than Bax in activated T cell death[33], at least in the initiation of the process. The differential expression and functions of Bak and Bax in various environments in which cells are dying, along with our observations here, indicate that they have different physiological functions in apoptosis. Bax

expression is induced at a later time point (48 h) than is Bak expression, which is upregulated in resting and early stages of the activation of Opn-knockout T cells. That observation can explain the biphasic characteristic of the survival curve of activated T cells in our results. That is, higher expression of Bim and Bak in resting Opn-knockout cells contributes to enhanced and accelerated death of Opn-knockout cells in an early stage, and the subsequent induction of Bax in Opn-knockout cells contributes to cell death at a later stage. The greater abundance of Bax at 48 h in Opn-knockout than in Opn-wild-type cells may compensate for the lower abundance of induction of Bim and Bak in the same time period. Our kinetic studies have indicated that Opn-knockout T cells are predisposed to death in association with increased abundance of Bim and Bak in the resting stage. Our findings further indicate that this enhanced cell death at a later stage would result in less survival of activated autoreactive T cells *in vivo* with resolution of relapses of EAE.

The Opn-induced signaling pathway, resulting in increased survival of activated T cells, seems to influence the caspase-independent death pathway, whereas the Fas-mediated death pathway is not affected by Opn. Consistent with our results, it has been reported that costimulation through $\alpha_v\beta_3$ integrin by Opn *in vitro* mediates IL-2 secretion without inducing programmed cell death of T cells[48] and, furthermore, studies of cardiac fibroblasts and human peripheral blood mononuclear cells have shown that the Opn effect is independent of caspase pathways and of Fas-mediated pathways, respectively[9]. Collectively, our findings emphasize the potential importance of cytokine-mediated regulation of the cell-autonomous death pathway in T cells.

Opn is known to have pleiotropic activities. We have not entirely excluded the possibility that other functions of Opn, such as its capacity to act as a chemoattractant and to induce proinflammatory functions, augment the prosurvival function of Opn suggested here, and together could augment the rapid progression of EAE. The modulation of NF-κB by Opn, for example, could modulate those proinflammatory functions and mediate cell death as well.

There is indirect evidence for involvement of Opn in spontaneous recovery from autoimmune diseases in other models. Notable are studies of mice with 'knockout' of CD44 variant exon 7 (CD44 v7), one of the receptors for Opn. CD44 v7–deficient mice and CD44 v6– and CD44 v7–deficient double-transgenic mice show complete spontaneous recovery from experimental colitis[49] similar to the spontaneous recovery of Opn-knockout mice from EAE. Experimental colitis is an animal model of human inflammatory bowel disease, such as Crohn disease, in which Opn is also upregulated[10]. More notably, increased cell death of leukocytes is found in inflamed regions of bowel in mice with *Cd44* deletions[49]. Hence, Opn receptor deficiency results in increased cell death of leukocytes that infiltrate the target tissue and results in spontaneous recovery in tissue-specific autoimmune diseases such as EAE and experimental colitis.

Our findings have not only demonstrated the inhibitory function of Opn in activated T cell death and its underlying mechanisms but have also shown the function of Opn in the relapse and progression of an autoimmune disease. Our study may provide new insights into the regulation of T cell viability, as they support the idea of an additional 'layer of protection' in the immune system for mitigating autoimmune attacks. Furthermore, our results provide a potential therapeutic target for the treatment of perplexing autoimmune relapses, presumably applicable to many autoimmune diseases.

METHODS

Mice. Female SJL/J, C57BL/6 and *Rag1*$^{-/-}$ mice 8–12 weeks old were purchased from the Jackson Laboratory. Mice transgenic for the myelin basic protein Ac 1-11 TCR (called 'MBP TCR–transgenic mice' here) have been described[50]. Opn-knockout and Opn-wild-type mice on a 129–C57BL/6 mixed background have been described[4]. Opn-knockout mice on a C57BL/6 background (backcrossed 11 generations) were used, and for Opn-wild-type mice on a C57BL/6 background, wild-type C57BL/6 mice were used as control (purchased from the Jackson Laboratory). MOG-specific TCR–transgenic mice (2D2) were backcrossed onto the C57BL/6 background[23]. All animal protocols were approved by the Division of Comparative Medicine at Stanford University and the Committee of Animal Research at the University of California San Francisco, in accordance with National Institutes of Health guidelines.

Peptides. MOG peptide (amino acids 35–55; MEVGWYRSPFSRVVHLYRNGK) and the peptide of proteolipid protein (amino acids 139–151; HCLGKWLGHPDKF) were synthesized on a peptide synthesizer (model 9050; MilliGen) by standard 9-fluorenylmethoxycarbonyl chemistry and were purified by high-performance liquid chromatography. Amino acid sequences were confirmed by amino acid analysis and mass spectrometry. The purity of each peptide was over 95%.

Induction of EAE. For SJL/J mice, EAE was induced with the peptide of proteolipid protein (amino acids 139–151). For C57BL/6 mice, Opn-knockout mice, Opn-wild-type mice and MOG-specific TCR–transgenic mice, EAE was induced by immunization with 100 μg of MOG peptide. All peptides were dissolved in complete Freund's adjuvant containing 4 mg/ml of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories) as described[4,50]. On the day of immunization and 48 h later, mice were injected intravenously with 50 ng of *Bordetella pertussis* toxin in PBS. Mice were examined daily for clinical signs of EAE and were assigned scores as follows: 0, no paralysis; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, hindlimb and forelimb paralysis; and 5, moribund or dead. For each mouse, remission was defined as a decrease in the score of at least one point for at least 2 consecutive days. For assessment of optic neuritis, MOG-specific TCR–transgenic mice (which can develop spontaneous optic neuritis) were examined daily for clinical signs, including eyelid redness and swelling as well as tearing and atrophy of the eye.

Administration of rOpn *in vivo*. Opn-knockout mice with EAE were treated by injection into the tail vein of 5 μg of carrier-free mouse rOpn (R&D Systems) suspended in 100 μl PBS. The residual endotoxin concentration in mouse rOpn was assessed by the limulus amoebocyte lysate test (Sigma); it was less than 0.003 endotoxin units per microgram of rOpn. PBS or lipopolysaccharide (0.5 ng per mouse) was administered intravenously as a control. Daily treatment began either on the same day of EAE induction or during the first remission of each mouse.

TUNEL assay of tissue sections and histopathology. Mice with EAE were killed 8–10 d after disease onset. Brains and spinal cords were fixed in 10% (volume/volume) saline-buffered formalin and were embedded in paraffin. TUNEL-positive cells were detected in deparaffinized sections with the Apoptag Plus Detection Kit (Chemicon International) according to the manufacturer's instructions. Sections stained with 3,3′-diaminobenzidine were fixed with 10% (volume/volume) saline-buffered formalin and were counterstained with hematoxylin. TUNEL-positive cells were counted by a researcher 'blinded' to the treatment status of the mouse.

TUNEL assay of peripheral lymphocytes. The In Situ Cell Death Detection Kit (Roche Molecular Biochemicals) was used according to the manufacturer's instructions for the TUNEL assay to detect apoptosis. Lymph node cells and splenocytes were stimulated and cultured for various time periods. In some cases, 100 mM z-VAD-fmk (BD PharMingen) was added to the culture. Cells were fixed for 1 h at 25 °C in 2% (weight/volume) paraformaldehyde and then were made permeable for 2 min at 0 °C in 0.1% (weight/volume) Triton-X100 in 0.1% (weight/volume) sodium citrate. Samples were then incubated for 1 h at 37 °C in the dark in a TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-conjugated dUTP to label DNA strand breaks. The fluorescence of cells carrying DNA labeled with FITC-dUTP (TUNEL-positive cells) was analyzed by flow cytometry.

CFSE labeling. Single-cell suspensions made from spleens and lymph nodes or from column-purified T cells were suspended at a density of 4×10^7 cells per ml in PBS containing 5% (volume/volume) FCS. CFSE (Molecular Probes) diluted in PBS containing 5% (volume/volume) FCS was added to an equal volume of prewarmed cell suspension at a final concentration of 5 μM and the suspension was mixed rapidly. Cells were incubated for 15 min at 37 °C and then were centrifuged (300*g*) for 5 min at 25 °C. The pellet was resuspended in the culture medium (described above; T cell activation and proliferation assay) and were incubated for an additional 30 min at 37 °C. At the end of the incubation, cells were washed three times in PBS containing 5% (volume/volume) FCS and were resuspended in the culture medium.

T cell activation. Splenocytes and lymph node cells were isolated from Opn-wild-type, Opn-knockout or MBP TCR–transgenic mice and were resuspended in culture medium consisting of RPMI 1640 medium supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-mercaptoethanol (50 μM) and either 10% (volume/volume) FCS for MBP TCR–transgenic cells or 1% (volume/volume) autologous normal mouse serum for Opn-wild-type and Opn-knockout cells. T cells isolated from spleen and lymph nodes were stimulated with concanavalin A (2 μg/ml) and were cultured for various time periods. For stimulation of T cells with anti-CD3 and anti-CD28, lymph node cells (2×10^6 cells/ml) or $CD3^+$ T cells (2×10^6 cells/ml) purified by negative selection (columns from R&D Systems) were stimulated in six-well plates coated with 5 μg/ml of anti-CD3 (clone 145-2C11; BD Biosciences) and 5 μg/ml of anti-CD28 (clone 37.51; BD Biosciences) or with 5 μg/ml of anti-CD3 alone.

Flow cytometry. A standard protocol was used for immunofluorescence staining of cells for flow cytometry. Cells (5×10^5 to 10×10^5) were suspended in flow cytometry buffer (2% (volume/volume) FCS in PBS) and were stained for 20 min at 25 °C. Fluorochrome-conjugated monoclonal antibodies (anti-CD4 and anti-CD8 conjugated to fluorescein isothiocyanate, phycoerythrin or peridinine chlorophyll protein; anti-CD3ε conjugated to fluorescein isothiocyanate or phycoerythrin; and anti–TCR $V_\beta 8$ conjugated to phycoerythrin) were purchased from BD PharMingen. For biotinylated antibodies (anti–TCR $V_\beta 6$ and anti–TCR $V_\beta 12$), fluorescein isothiocyanate– or phycoerythrin-conjugated streptavidin was used (BD PharMingen). Stained cells were washed three times and were analyzed with a FACScan and CellQuest software (Becton Dickinson) and FlowJo software (TreeStar). For assessment of cell viability, 1 μg/ml of propidium iodide was added. A total of 5×10^4 to 1×10^5 events were analyzed.

Immunoblot analysis and NF-κB activation assay. Nuclear and mitochondrial fractions were obtained by differential centrifugation. Mouse lymph node cells and splenocytes (5×10^6 cells) were washed with ice-cold PBS and were resuspended in 200 μl of ice-cold isotonic homogenization buffer (250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride and 10 mM Tris-HCl, pH 7.4) containing a protease inhibitor 'cocktail' (Roche). Cells were broken by 80 strokes with a prechilled Dounce homogenizer, then the homogenates were 'spun down' for 5 min at 30*g* for the removal of unbroken cells. Nuclei were pelleted by centrifugation of the supernatant for 10 min at 750*g*. For the mitochondrial fraction, supernatants were centrifuged again for 20 min at 14,000*g*. Pellets from the nuclei were washed three times with homogenization buffer containing 0.01% (weight/volume) Nonidet-P40. Lysates of 1×10^6 cells were used for each lane for SDS-PAGE, and the ECL detection system (Amersham BioSciences) was used for immunoblot analysis. Antibodies for immunoblot analysis were purchased from the following sources: anti–histone H3 (9715), anti-Foxo3a (9467), and antibodies to phosphorylated PDK1 (3061), PTEN (9551) and Foxo3a (9465), Cell Signaling; anti-Bim (559685), BD PharMingen; anti-Bak (06-536) and anti-Bax (06-499), Upstate Biotechnologies; anti-β-actin (A2066), Sigma; and anti-AIF (H-300; sc-5586), Santa Cruz Biotechnologies. Proteins detected on immunoblots were quantified by measurement of band intensity using ImageJ software (National Institutes of Health). The band intensity of each protein was normalized to that of β-actin in the same blot. For the NF-κB activation assay, nuclear extracts from T cells were isolated with the Transfactor Extraction Kit (Clontech Laboratories) according to the manufacturer's instructions. The concentration of proteins for each sample was determined with the BCA assay kit (Pierce Chemical). The NF-κB TransFactor enzyme-linked immunosorbent assay–based kit (Clontech Laboratories) was used for DNA-binding assays for NF-κB p65 and p50.

Statistical analysis. Statistical significance was analyzed with a one-way multiple-range analysis of variance (ANOVA) for multiple comparisons or the Fisher exact-probability test (one-tailed). A P value of less than 0.05 was considered significant.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank G.R. Crabtree, P.P. Jones, P.J. Utz, T.W. Mak (University of Toronto) and D.R. Green (St. Jude Children's Research Hospital) for discussions; J.H. Kim, M.J. Eaton and C.K. Chung for technical assistance; C.J. Shabrami and M.M. Winslow for critical review of the manuscript; S. Rittling and D.T. Denhardt (Rutgers University) for Opn-knockout mice; and V. Kuchroo (Harvard University) for 2D2 mice. Supported by the National Health Institutes, the National Multiple Sclerosis Society, the Phil N. Allen Trust, a Stanford Graduate Fellowship (E.M.H.), a Korean Government Overseas Scholarship (E.M.H.) and a National Multiple Sclerosis Society Career Transitional Award (S.Y.).

AUTHOR CONTRIBUTIONS

E.M.H. and S.Y. designed and did the experiments and prepared the manuscript; M.E.H. assisted with cell fractionation; S.Y.Z. assisted in assigning scores to mice with EAE; R.A.S. contributed to histology; and L.S. supervised all studies and preparation of the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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