Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy

GILA MOALEM[1,2], RAYA LEIBOWITZ-AMIT[1], ETI YOLES[1], FELIX MOR[2], IRUN R. COHEN[2] & MICHAL SCHWARTZ[1]

[1]*Department of Neurobiology,* [2]*Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel*
Correspondence should be addressed to M.S.; email: bnschwar@weizmann.weizmann.ac.il
G.M. & R.L.-A. contributed equally to the work.

Autoimmunity to antigens of the central nervous system is usually considered detrimental. T cells specific to a central nervous system self antigen, such as myelin basic protein, can indeed induce experimental autoimmune encephalomyelitis, but such T cells may nevertheless appear in the blood of healthy individuals. We show here that autoimmune T cells specific to myelin basic protein can protect injured central nervous system neurons from secondary degeneration. After a partial crush injury of the optic nerve, rats injected with activated anti-myelin basic protein T cells retained approximately 300% more retinal ganglion cells with functionally intact axons than did rats injected with activated T cells specific for other antigens. Electrophysiological analysis confirmed this finding and suggested that the neuroprotection could result from a transient reduction in energy requirements owing to a transient reduction in nerve activity. These findings indicate that T-cell autoimmunity in the central nervous system, under certain circumstances, can exert a beneficial effect by protecting injured neurons from the spread of damage.

Maintenance of central nervous system (CNS) integrity is a complex balancing act in which compromises are struck with the immune system. In most tissues, the immune system plays an essential part in protection, repair and healing. In the CNS, because of its unique immune privilege, immunological reactions are relatively limited[1,2]. A growing body of evidence indicates that the failure of the mammalian CNS to achieve functional recovery after injury is a reflection of an ineffective 'dialog' between the damaged tissue and the immune system. For example, the restricted communication between the CNS and blood-borne macrophages affects the capacity of axotomized axons to regrow, and transplantation of activated macrophages can promote CNS regrowth[3,4]. Here we have extended the study of CNS maintenance to T cells.

Activated T cells have been shown to enter the CNS parenchyma, irrespective of their antigen specificity, but only T cells capable of reacting with a CNS antigen seem to persist there[5]. T cells reactive to antigens of CNS white matter, such as myelin basic protein (MBP), can induce the paralytic disease experimental autoimmune encephalomyelitis (EAE) within several days of their inoculation into naive recipient rats[6]. Anti-MBP T cells may also be involved in the human disease multiple sclerosis[7,8]. However, despite their pathogenic potential, anti-MBP T-cell clones are present in the immune systems of healthy subjects[9–12]. Activated T cells, which normally patrol the intact CNS, transiently accumulate at sites of CNS white matter lesions[13]. These observations prompted us to study whether the T cells that accumulate after axonal injury exert a beneficial or a deleterious effect on the damaged CNS.

A catastrophic consequence of CNS injury is that the primary damage is often compounded by the gradual secondary loss of adjacent neurons that apparently were undamaged, or only marginally damaged, by the initial injury[14–16]. The primary lesion causes changes in extracellular ion concentrations, elevation of amounts of free radicals, release of neurotransmitters, depletion of growth factors, and local inflammation. These changes trigger a cascade of destructive events in the adjacent neurons that initially escaped the primary injury[17–19]. This secondary damage is mediated by activation of voltage-dependent or agonist-gated channels, ion leaks, activation of calcium-dependent enzymes such as proteases, lipases and nucleases, mitochondrial dysfunction and energy depletion, culminating in neuronal cell death[20–23]. The widespread loss of neurons beyond the loss caused directly by the primary injury has been called 'secondary degeneration'.

We have developed a model for studying secondary degeneration, based on a partial crush injury of the rat optic nerve[23–25]. Here we demonstrate morphologically that activated T cells specific to the CNS self antigen MBP can reduce the secondary degeneration of neurons after a primary crush injury. T cells specific to an epitope of a different self antigen, the 60-kDa heat shock protein (hsp60), or to the foreign antigen ovalbumin (OVA) did not protect neurons against secondary degeneration, although these T cells did 'home' to the site of optic nerve injury. Electrophysiological analysis confirmed the neuroprotective effect of the anti-MBP T cells. The observed neuroprotection was preceded by a transient reduction in nerve conduction. These findings indicate that the anti-MBP T cells might reduce injury-induced secondary damage by inducing a resting state in the damaged nerve, thereby reducing its energy demands and enhancing its ability to cope with the stress resulting from the injury.

Presence of T cells in the injured optic nerve

Passively transferred, activated T cells accumulate at a site of CNS injury, independent of their antigen specificity[13].

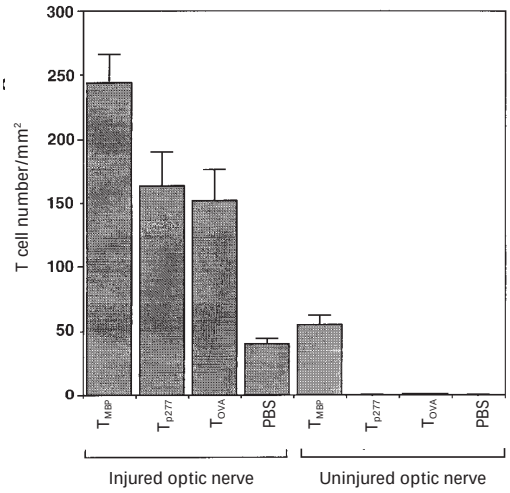

Fig. 1 T-cell presence in injured optic nerve 1 week after injury. Adult Lewis rats were injected with activated T cells of the anti-MBP (T_{MBP}), anti-OVA (T_{OVA}), or anti-p277 (T_{p277}) lines, or with PBS, immediately after unilateral crush injury of the optic nerve. Seven days later, both the injured and uninjured optic nerves were removed, cryosectioned and analyzed immunohistochemically for the presence of immunolabeled T cells. T cells were counted at the site of injury and at randomly selected areas in the uninjured optic nerves. The histogram shows the mean number of T cells per mm^2 ± s.e.m., counted in two to three sections of each nerve. Each group contained three to four rats. The number of T cells was considerably higher in injured nerves of rats injected with anti-MBP, anti-OVA or anti-p277 T cells; statistical analysis (one-way ANOVA) showed significant differences between T cell numbers of injured optic nerves of rats injected with anti-MBP, anti-OVA, or anti-p277 T cells and injured optic nerves of rats injected with PBS ($P < 0.001$); and between injured optic nerves and uninjured optic nerves of rats injected with anti-MBP, anti-OVA, or anti-p277 T cells ($P < 0.001$).

Therefore, we analyzed crush-injured optic nerves for the presence of T cells after systemic injection of various T-cell lines. Autoimmune T cells specific to MBP, T cells specific to the non-self antigen OVA, or T cells specific to peptide 277 (p277) of hsp60 (a self antigen not restricted to the CNS) were activated with their respective antigens for 3 days, and then injected (1×10^7 cells) intraperitoneally into rats immediately after unilateral optic nerve injury. Control rats were injected intraperitoneally with phosphate-buffered saline (PBS). Seven days after injury, the optic nerves were excised, cryosectioned and analyzed immunohistochemically for the presence of T cells. No T cells could be detected in the uninjured optic nerves of PBS-injected rats (Fig. 1). Anti-MBP T cells are known to 'home' to intact CNS white matter[26], and small numbers of T cells were indeed observed in the uninjured optic nerves of rats injected with anti-MBP T cells, but not in rats injected with anti-OVA or anti-p277 T cells. Crush injury of the optic nerve in PBS-injected control rats was accompanied by the presence of a small number of endogenous T cells at the injury site, possibly reflecting a response to self antigens triggered by the injury[27]. The number of T cells in the injured optic nerve in rats injected with PBS was 16% to 25% of the number in rats injected with anti-OVA, anti-p277 or anti-MBP T cells. These observations confirmed our previous finding that axonal injury in the CNS is accompanied by the accumulation of endogenous T cells, and that this accumulation is significantly augmented by systemic injection of activated T cells[13]. 'Homing' of exogenous T cells to the site of optic nerve injury can be demonstrated by prelabeling the injected T cells[13].

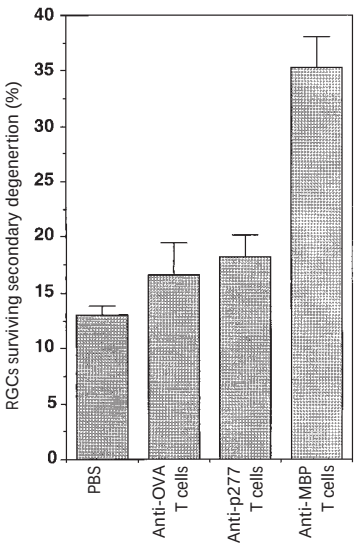

Fig. 2 T cells specific to MBP, but not to OVA or p277 of hsp60, protect neurons from secondary degeneration. Immediately after optic nerve injury, rats were injected with anti-MBP, anti-OVA or anti-p277 T cells, or with PBS. The neurotracer dye 4-Di-10-Asp was applied to optic nerves distal to the site of the injury, immediately after injury (for assessment of primary damage) or 2 weeks later (for assessment of secondary degeneration). Five days after dye application, the retinas were excised and flat-mounted. Labeled retinal ganglion cells (RGCs) from three to five randomly selected fields in each retina (all located at approximately the same distance from the optic disk) were counted by fluorescence microscopy. RGC survival in each group of injured nerves was expressed as the percentage of the total number of neurons spared after the primary injury (42% of axons remained undamaged after the primary injury). The neuroprotective effect of anti-MBP T cells compared with that of PBS was significant ($P < 0.001$, one-way ANOVA). Anti-OVA T cells or anti-p277 T cells did not differ significantly from PBS in their effect on the protection of neurons that had escaped the primary injury ($P > 0.05$, one-way ANOVA). The results are a summary of five experiments. Each group contained five to ten rats.

Neuroprotection by autoimmune anti-MBP T cells

Morphological analyses were done to assess the effect of the T cells on the response of the nerve to injury, and specifically on secondary degeneration. Immediately after optic nerve injury, rats were injected intraperitoneally with PBS or with 1×10^7 activated T cells of the various cell lines. The degree of primary damage to the optic nerve axons and their attached retinal ganglion cells (RGCs) was measured by injecting the dye 4-Di-10-Asp distal to the site of the lesion immediately after the injury. A time lapse of 2 weeks between a moderate crush injury and dye application is optimal for demonstrating the number of still-viable labeled neurons as a measure of secondary degeneration, and as the response of secondary degeneration to treatment. Therefore, secondary degeneration was quantified here by injecting the dye immediately or 2 weeks after the primary injury, and calculating the additional loss of RGCs between the first and the second injections of the dye. The percentage of RGCs that had survived secondary degeneration was then calculated. The percentage of labeled RGCs (reflecting still-viable axons) was significantly greater in the retinas of the rats injected with anti-MBP T cells than in the retinas of the PBS-injected control rats (Fig. 2). In contrast, the percentage of labeled RGCs in the retinas of the rats injected with anti-OVA or anti-p277 T cells was not significantly greater than those in the control retinas. Thus, although the three T-cell lines accumulated at the site of injury, only the MBP-specific autoimmune T cells had a substantial effect in limiting the extent of secondary degeneration. Photomicrographs show the labeled RGCs of injured optic nerves of rats injected with PBS, anti-

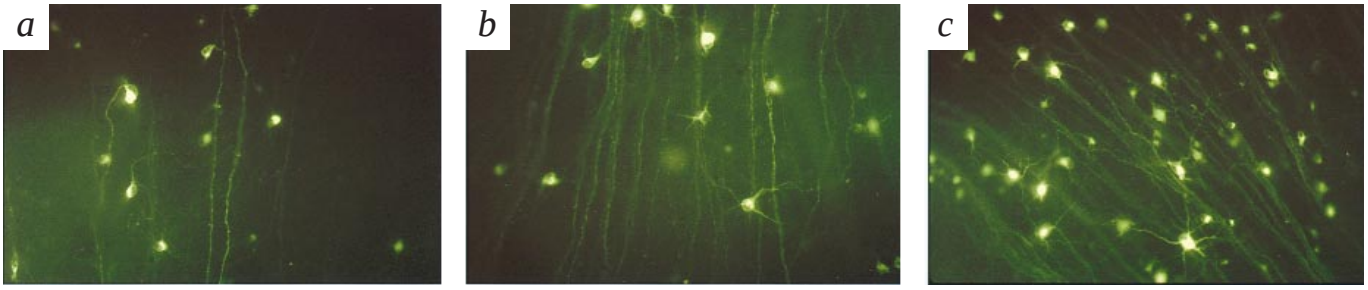

Fig. 3 Photomicrographs of retrogradely labeled retinas of injured optic nerves of rats. Immediately after unilateral crush injury of their optic nerves, rats were injected with PBS (***a***) or with activated anti-p277 T cells (***b***) or activated anti-MBP T cells (***c***). Two weeks later, the neurotracer dye 4-Di-10-Asp was applied to the optic nerves, distal to the site of injury. After 5 days, the retinas were excised and flat-mounted. Labeled (surviving) RGCs, located at approximately the same distance from the optic disk in each retina, were photographed.

p277 T cells or anti-MBP T cells (Fig. 3).

The functional autoimmunity of the injected anti-MBP T cells was demonstrated by the development of transient EAE in the recipients of these cells. The course and severity of the EAE was not affected by the presence of the optic nerve crush injury (Fig. 4*a*). Moreover, the administration of anti-MBP T cells in rats that had not suffered an optic nerve crush did not affect the numbers of RGCs (Fig. 4*b*). Thus, contracting EAE in the absence of a nerve crush did not cause any change in the numbers of viable RGCs or optic nerve axons.

To determine whether the neuroprotective effect of the anti-MBP T cells is correlated with their virulence, we examined the effect of T cells reactive to a 'cryptic' epitope of MBP, the peptide 51–70 (p51–70). 'Cryptic' epitopes activate specific T cells after an animal is immunized with the particular peptide, but not with the whole antigen[28]. The T-cell line reactive to the whole MBP and the T-cell line reactive to the cryptic epitope p51–70 were compared for the severity of the EAE they induced, and for their effects on secondary degeneration. In rats injected with the T-cell line reactive to the cryptic epitope, disease severity (as manifested by the maximal EAE score) was significantly lower than that in rats injected with the T-cell line reactive to the whole protein (Table 1). Whereas anti-MBP T cells caused clinical paralysis of the limbs, rats injected with the anti-p51-70 T cells developed only tail atony, not hind limb paralysis, and almost none showed weakness of the hind limbs. Despite this difference in EAE severity, the neuroprotective effect of the less virulent (anti-p51–70) T cells was similar to that of the more virulent (anti-MBP) T cells (Fig. 5). The percentage of RGCs surviving secondary degeneration in the retinas of rats injected with either of the lines was significantly higher than in the retinas of the PBS-injected rats. Thus, there was no correlation between the neuroprotective effect of the autoimmune T cells and their virulence. It is possible that the anti-p51–70 T cells encounter little antigen in the intact CNS, and therefore cause only mild EAE. Their target antigen may however become more available after injury, enabling these T cells to exert a neuroprotective effect.

Table 1 Anti-MBP and anti-p51-70 T cells vary in pathogenicity

T cell line	Clinical EAE	Mean max. score
Whole MBP	Moderate to severe	2.00 ± 0.25
p51–70 of MBP	Mild	0.70 ± 0.2

Immediately after optic nerve crush injury, Lewis rats were injected with activated anti-MBP T cells or anti-p51–70 T cells. The clinical course of EAE was evaluated according to the neurological paralysis scale. The mean maximal (max.) score ± s.e.m. was calculated as the average maximal score of all the diseased rats in each group. The table is a summary of nine experiments. Each group contained five to ten rats. Statistical analysis showed a significant difference between the mean maximal score of rats injected with anti-MBP T cells and that of rats injected with anti-p51–70 T cells ($P = 0.039$, Student's *t*-test).

To confirm the neuroprotective effect of the anti-MBP T cells, we did electrophysiological studies. Immediately after optic nerve injury, the rats were injected intraperitoneally with PBS or with 1×10^7 activated anti-MBP or anti-OVA T cells. The optic nerves were excised 7, 11 or 14 days later and the compound action potentials (CAPs), a measure of nerve conduction, were recorded from the uninjured nerves and from the distal segments of the injured nerves. On day 14, the mean CAP amplitude of the distal segments recorded from the injured nerves obtained from from rats injected with the anti-MBP T cells was about 250% that of recorded from the PBS-injected contrtol rats (Fig. 6*a* and Table 2). As the distal segment of the injured nerve contains both axons that escaped the primary insult and injured axons that have not yet degenerated, the observed neuroprotective effect could reflect the rescue of spared neurons, or a delay of Wallerian degeneration of the injured neurons (which normally occurs in the distal stump), or both. No effect of the injected anti-MBP T cells on the mean CAP amplitudes of uninjured nerves was observed (Fig. 6*b*, Table 2). It is unlikely that the neuroprotective effect observed on day 14 could have been due to the regrowth of nerve fibers, as the time period was too short for this.

The strong neuroprotective effect of the anti-MBP T cells seen on day 14 was associated with a significantly decreased CAP amplitude recorded on day 7 (Table 2). The anti-MBP T cells manifested no substantial effect on the uninjured nerve on day 7, indicating that the reduction in electrophysiological activity observed in the injured nerve on day 7 might reflect the larger number of T cells present at the injury site relative to the uninjured nerve (Fig. 1). The observed reduction in CAP amplitude in the injured nerve on day 7 reflected a transient reduction in conduction, which may have imposed a transient resting state in the injured nerve. This transient effect had not only disappeared, but was even reversed by day 14 (Table 2). Early signs of the neuroprotective effect could already be detected on day 11 in the rats injected with anti-MBP T cells (data not shown). In rats injected with anti-OVA T cells, no reduction in CAP amplitude on day 7 could be detected in either the injured or the uninjured nerves, and no neuroprotective effect was observed on day 14 (Table 2). Thus, it seems that the early reduction in CAP

Table 2 Transient reduction in electrophysiological activity of the injured optic nerve induced by anti-MBP T cells, followed by a neuroprotective effect.

	Uninjured optic nerve		Injured optic nerve	
	Day 7	Day 14	Day 7	Day 14
Ratio (%) T_{MBP}/PBS	89.9 ± 9.4 (*n* = 22)	101.2 ± 22.7 (*n* = 10)	63.8 ± 14.9* (*n* = 17)	243.1 ± 70.8** (*n* = 8)
Ratio (%) T_{OVA}/PBS	109.7 ± 13.2 (*n* = 11)	92.5 ± 12.6 (*n* = 3)	125.5 ± 24.4 (*n* = 11)	107.3 ± 38.9 (*n* = 4)

Immediately after optic nerve injury, rats were injected with PBS or with activated anti-MBP or anti-OVA T cells. After 7 or 14 days, the CAPs of injured and uninjured nerves were recorded. Ratios were calculated for uninjured nerves as (mean CAP of uninjured nerves from T cell-injected rats/mean CAP of uninjured nerves from PBS-injected rats) × 100, or for injured nerves as (mean CAP of injured nerves from T cell-injected rats/mean CAP of injured nerves from PBS-injected rats) × 100. The *P* value was calculated by comparing the logarithms of the normalized CAP amplitudes of nerves from PBS-injected rats and rats injected with T cells, using the unpaired Student's *t*-test. *, $P < 0.05$; **, $P < 0.01$.

and the late neuroprotection shown specifically by the anti-MBP T cells are related.

Discussion

The CNS is an immune-privileged site in which local immune responses are restricted[1,2] and in which the capacity for repair and recovery after injury is poor[29]. The highly specialized CNS must avoid the possibly destructive consequences of severe inflammation and autoimmune disease. However, immune reactions are required for healing and recovery after injury in the CNS, as in other tissues[30]. An accumulating body of evidence indicates that the failure of the CNS to recover from injury is related to its immune-privileged status. In particular, the resistance of the CNS to regeneration might be closely related to the restriction in the numbers of macrophages recruited and activated by the injured CNS (ref. 3). Here we have demonstrated that the administration of autoimmune anti-MBP T cells to rats with injured optic nerves, rather than aggravating the damage, can lead to a significant degree of protection from secondary degeneration. Thus, enhancement of the T-cell autoimmune response to a component of CNS myelin seemed to be beneficial in limiting the spread of secondary damage after partial injury of CNS axons.

Autoimmune responses directed against antigens of the CNS are usually regarded as detrimental. As an example, passive transfer of T-cell lines specific for MBP or proteolipid protein induces a paralytic disease, EAE[6,31]. We have shown here, however, using both morphological and electrophysiological techniques, that T-cell autoimmunity can mediate significant neuroprotection after CNS injury. The slight difference in therapeutic index obtained by these two techniques can be attributed to the fact that the former detects only axons that are still morphologically intact, whereas the latter, in which CAPs are recorded from the nerve segment distal to the injury site, might reflect the activities of both intact axons and injured axons that are still functioning.

CNS damage can activate latent autoimmunity to MBP: myelin-reactive antibodies are elevated after CNS injury[32,33], and T cells isolated from rats with spinal cord injury are capable of causing EAE in naive rats[27]. Therefore, it will be important to determine whether the endogenous T cells accumulating at the site of damaged optic nerve include anti-MBP clones. The results reported here using anti-MBP T cell lines indicate that endogenous anti-MBP T cells, if sufficiently numerous, might function in a similar way to protect damaged CNS tissue. The lack of correlation between the clinical pathogenicity of the autoimmune T cells and their neuroprotective effect indicates that a benign autoimmunity, achieved by non-encephalitogenic T cells specific to a cryptic antigen, might serve as an effective mechanism for neuroprotection. In the uninjured CNS, cryptic epitopes might not be readily accessible and, therefore, T cells to such epitopes might cause only mild, if any, autoimmune disease. After injury, however, cryptic epitopes might become available and the specific T cells could then be activated at the site of injury to exert their neuroprotective effect.

At present, it seems that neuroprotection is exerted only by the CNS-specific autoimmune T cells. The inefficiency of the anti-p277 T cells is noteworthy, given that the hsp60 molecule is expressed in injured tissues[34] and anti-hsp60 T cells have been isolated from EAE lesions[35]. Thus, not all autoimmune T cells can inhibit secondary degeneration, even if the target antigen is present in the CNS lesion. It is possible, however, that the p277 epitope of hsp60 is not strongly expressed in the injured optic nerve. We are now investigating the neuroprotective potential of T cells specific for other self antigens.

In addition to the question of specificity, the molecular mechanisms by which anti-MBP T cells protect the injured nerve from secondary degeneration of neurons need to be ex-

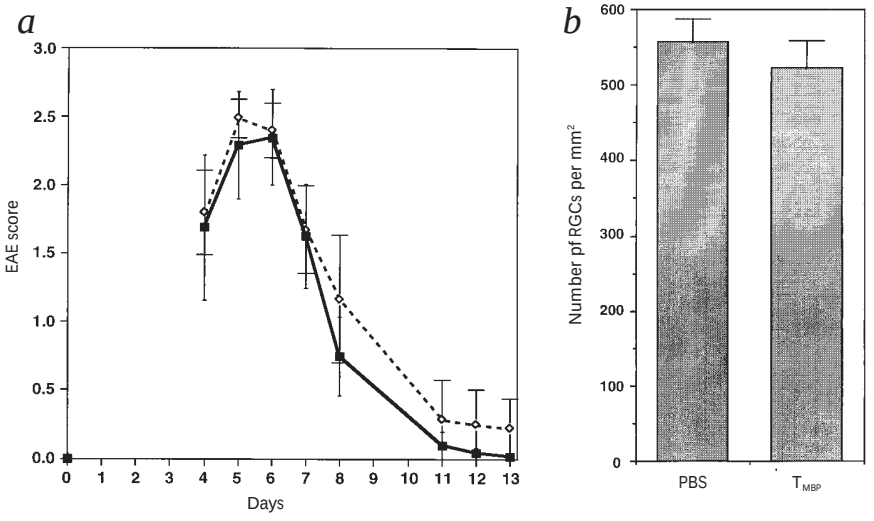

Fig. 4 ***a***, Clinical severity of EAE is not influenced by an optic nerve crush injury. Lewis rats, either uninjured (⊥) or immediately after optic nerve crush injury (⊣), were injected with activated anti-MBP T cells. EAE was evaluated according to a neurological paralysis scale. Data points represent means ± s.e.m. These results represent a summary of three experiments. Each group contained five to nine rats. ***b***, The number of RGCs in the uninjured optic nerve is not influenced by injection of anti-MBP T cells. Two weeks after the injection of anti-MBP T cells or PBS, 4-Di-10-Asp was applied to the optic nerves. After 5 days the retinas were excised and flat-mounted. Labeled RGCs from five fields (located at approximately the same distance from the optic disk) in each retina were counted and their average number per mm² was calculated. There was no difference in the number of labeled RGCs between rats injected with anti-MBP T cells (T_{MBP})and PBS-injected control rats.

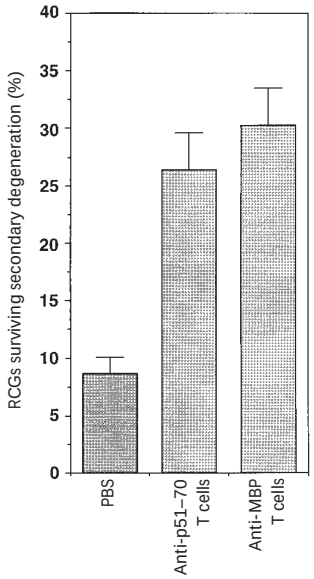

Fig. 5 T cells specific to p51–70 of MBP protect neurons from secondary degeneration. Immediately after optic nerve injury, rats were injected with anti-MBP T cells, anti-p51–70 T cells or PBS. The neurotracer dye 4-Di-10-Asp was applied to optic nerves distal to the site of the injury, immediately after injury (for assessment of primary damage) or 2 weeks later (for assessment of secondary degeneration). Five days after dye application, the retinas were excised and flat-mounted. Labeled retinal ganglion cells (RGCs) from three to five randomly selected fields in each retina (all located at approximately the same distance from the optic disk) were counted by fluorescence microscopy. RGC survival in each group of injured nerves was expressed as the percentage of the total number of neurons spared after the primary injury. Compared with that of PBS treatment, the neuroprotective effects of anti-MBP and anti-p51–70 T cells were significant ($P < 0.001$, one-way ANOVA).

plored further. Secondary degeneration results from metabolic insult, among other factors[20,21,23,36]. Given our electrophysiological findings here, the anti-MBP T cells might exert neuroprotection by causing a transient reduction in the nerve's electrophysiological activity. Putting the damaged nerve to rest, even transiently, could reduce the nerve's metabolic oxygen and glucose requirements and prevent energy depletion, thus helping to preserve neuronal viability. The mechanism proposed here is reminiscent of the neuroprotection obtained by therapeutic hypothermia[37]. Our finding here agrees with earlier work showing that anti-MBP T cells could reversibly block signal conduction in the isolated rat optic nerve *in vitro*[38], and indicating that no invading cells other than T cells are required for the observed reduction in electrophysiological activity of the nerve. The exposure of myelin at the site of the crush might activate MBP-specific T cells to secrete molecules that put the injured nerve to rest, whereas T cells specific for other antigens might not be activated because of inadequate antigen recognition. The anti-MBP T cells may act directly on neurons or on glial cells (for example, astrocytes), which can indirectly mediate neuronal dysfunction after immunological activation[39].

There is evidence that cytokines can directly affect the electrophysiological functions of neurons and glial cells. Cytokines can induce a reduction in neuronal excitability, for example by increasing inactivation of the Na^+ current[39]. Inhibition of neuronal excitability by T-cell cytokines may contribute to the CAP reduction we observed in the optic nerves of the rats injected with the anti-MBP T cells. It is unlikely that this reduction in electrophysiological activity is due to demyelination, as EAE adoptively transferred to Lewis rats by anti-MBP T cells is typically a brief and monophasic disease with sparse demyelination[40].

Although the mechanism proposed here seems most promising, alternative explanations cannot be excluded. Growth factors secreted by anti-MBP T cells in the tissue might protect the injured CNS. $CD4^+$ T cells were indeed found to synthesize and release biologically active nerve growth factor[41–43]. Growth factors can attenuate the elevation of levels of Ca^{++} and free radicals otherwise 'triggered' in neurons by damage. Thus, the benefit provided by the anti-MBP T cells could be mediated by several actions, working together.

Our findings here indicate that the activation of specific autoimmunity in the CNS might not always be detrimental, but could, under certain circumstances, have a physiological role in protecting the damaged CNS. Beneficial autoimmunity is functionally distinguishable from autoimmune disease. If autoimmunity to a particular protein or to a selected epitope can be advantageous, we might have an explanation for the wide prevalence of well-regulated autoimmunity naturally directed to a particular set of self antigens, a phenomenon that has been called the 'immunological homunculus'[44]. The immunological dominance of the main self antigens constituting the homunculus is encoded by naturally autoimmune T cells and B cells and their anti-idiotypic regulatory cells[45]. Indeed, the idea that the immune system's primary goal is to discriminate between self and non-self was recently called into question. Our results are consistent with the idea that a primary function of the immune system is to maintain the body by receiving signals from an extended network of body tissues, without necessarily ignoring self antigens. Thus, the immune system can be activated to deal with tissue damage, rather than exclusively with the danger associated with pathogens[46]. The fact that endogenous autoimmune T cells apparently do not function optimally in protecting neurons after CNS injury may reflect evolutionary constraints derived from the special needs of the highly specialized CNS, which restrict immune reactions. If, however, autoimmunity to particular injury-exposed epitopes can be selectively augmented, it might be possible to achieve neuroprotection without the threat of autoimmune disease.

Our findings here not only demonstrate the role that T cells

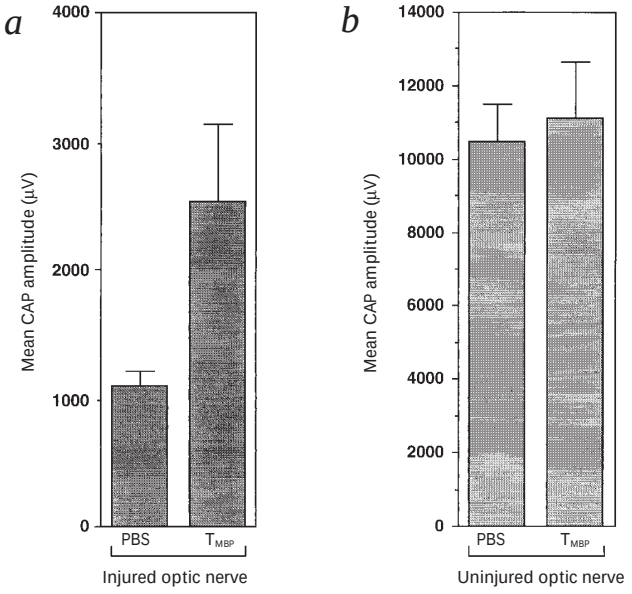

Fig. 6 Anti-MBP T cells increase the CAP amplitude of injured optic nerves. Immediately after optic nerve injury, rats were injected with either PBS or activated anti-MBP T cells (T_{MBP}). Two weeks later, the CAPs of injured (***a***) and uninjured (***b***) nerves were recorded. There were no significant differences in mean CAP amplitudes between uninjured nerves obtained from PBS-injected and T cell-injected rats ($n = 8$; $P = 0.8$, Student's *t*-test). The neuroprotective effect of anti-MBP T cells (relative to PBS) on the injured nerve on day 14 after injury was significant ($n = 8$; $P < 0.01$, Student's *t*-test).

might play in nerve recovery, but also further substantiate the idea that natural autoimmunity can be benign[44] and may even function as a protective mechanism.

Methods

Animals. Inbred female adult Lewis rats (8–12 weeks old) were supplied by the Animal Breeding Center of The Weizmann Institute of Science. The rats were housed in a light- and temperature-controlled room and matched for age in each experiment.

Antigens. MBP from the spinal cords of guinea pigs was prepared as described[47]. OVA was purchased from Sigma. The p51–70 of the rat 18.5-kDa isoform of MBP (sequence APKRGSGKDSH—TRTTHYG) and the p277 of the human hsp60 (sequence VLGGGCALLRCPALDSLTPANED)(ref. 48) were synthesized using the 9-fluorenylmethoxycarbonyl technique with an automatic multiple peptide synthesizer (AMS 422; ABIMED, Langenfeld, Germany). The purity of the peptides was analyzed by HPLC and amino-acid composition.

T-cell lines. T-cell lines were generated from draining lymph node cells obtained from Lewis rats immunized with the antigens above[49]. The antigen was dissolved in PBS (1 mg/ml) and emulsified with an equal volume of incomplete Freund's adjuvant (Difco Laboratories, Detroit, Michigan) supplemented with 4 mg/ml *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, Michigan). Ten days after the antigen was injected into the rats' hind foot pads in 0.1 ml of the emulsion, the rats were killed and draining lymph nodes were surgically removed and dissociated. The cells were washed and activated with the antigen (10 μg/ml) in proliferation medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), nonessential amino acids (1 ml/100 ml) and autologous serum 1% (volume/volume). After incubation for 72 h at 37 °C, 90% relative humidity and 7% CO_2, the cells were transferred to propagation medium consisting of DMEM, L-glutamine, 2-mercaptoethanol, sodium pyruvate, nonessential amino acids and antibiotics in the same concentrations as above, with the addition of 10% fetal calf serum (FCS) (volume/volume) and 10% T-cell growth factor derived from the supernatant of concanavalin A-stimulated spleen cells[50]. Cells were grown in propagation medium for 4–10 days before being re-stimulated with their antigen (10 μg/ml) in the presence of irradiated (2000 rad) thymus cells (10^7 cells/ml) in proliferation medium. The T-cell lines were expanded by repeated stimulation and propagation.

Crush injury of optic nerve. Crush injury of the optic nerve was done as described[51]. Rats were deeply anesthetized by intraperitoneal injection of xylazine, (10 mg/kg; Vitamed, Bat-Yam, Israel) and ketamine (50 mg/kg; Fort Dodge Laboratories, Fort Dodge, Iowa). Using a binocular operating microscope, a lateral canthotomy was done in the right eye and the conjunctiva was incised lateral to the cornea. After separation of the refractor bulbi muscles, the optic nerve was exposed intraorbitally by blunt dissection. Using calibrated cross-action forceps, the optic nerve was subjected to a moderate crush injury 1–2 mm from the eye. The uninjured contralateral nerve was left undisturbed.

Immunocytochemistry of T cells. Longitudinal cryosections of the excised nerves (20 μm in thickness) were picked up onto gelatin-coated glass slides and frozen until preparation for fluorescence staining. Sections were fixed in ethanol for 10 min at room temperature, washed twice in double-distilled water and incubated for 3 min in PBS containing 0.05% polyoxyethylene-sorbitan monolaurate (Tween-20). Sections were then incubated for 1 h at room temperature with mouse monoclonal antibody to rat T-cell receptor[52] (donated by B. Reizis) diluted in PBS containing 3% FCS and 2% bovine serum albumin. The sections were washed three times with PBS containing 0.05% Tween-20 and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (with minimal cross-reaction to rat, human, bovine and horse serum proteins)(Jackson ImmunoResearch, West Grove, Pennsylvania), for 1 h at room temperature. The sections were washed with PBS containing Tween-20 and treated with glycerol containing 1,4-diazobicyclo-(2,2,2) octane to inhibit quenching of fluorescence. Sections were viewed with a Zeiss microscope and cells were counted. Staining in the absence of first antibody was negative.

Retrograde labeling and measurement of primary damage and secondary degeneration. Primary damage of the optic nerve axons and their attached RGCs was measured after the immediate post-injury application of the fluorescent lipophilic dye 4-(4-(didecylamino)styryl)-n-methylpyridinium iodide (4-Di-10-Asp) (Molecular Probes Europe BV, Leiden, Netherland) distal to the site of injury. Only axons that are intact are capable of transporting the dye back to their cell bodies; therefore, the number of labeled cell bodies is a measure of the number of axons that survived the primary damage. Secondary degeneration was also measured by application of the dye distal to the injury site, but 2 weeks after the primary lesion was inflicted. Application of the neurotracer dye distal to the site of the primary crush after 2 weeks ensures that only axons that survived both the primary damage and the secondary degeneration will be counted. This approach makes it possible to differentiate between neurons that are still functionally intact and neurons in which the axons are injured but the cell bodies are still viable, as only those neurons whose fibers are morphologically intact can take up dye applied distally to the site of injury and transport it to their cell bodies. Using this method, the number of labeled ganglion cells reliably reflects the number of still-functioning neurons. Labeling and measurement were done by exposing the right optic nerve for a second time, again without damaging the retinal blood supply. Complete axotomy was done 1–2 mm from the distal border of the injury site and solid crystals (0.2–0.4 mm in diameter) of 4-Di-10-Asp were deposited at the site of the newly formed axotomy. Uninjured optic nerves were similarly labeled at approximately the same distance from the globe. Five days after dye application, the rats were killed. The retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution and examined for labeled ganglion cells by fluorescence microscopy. The percentage of RGCs surviving secondary degeneration was calculated using the following formula: (Number of spared neurons after secondary degeneration)/(Number of spared neurons after primary damage) × 100.

Electrophysiological recordings. Nerves were excised and their CAPs were recorded *in vitro* using the suction electrode experimental set-up described[53]. At different times after injury and injection of T cells or PBS, rats were killed by intraperitoneal injection of pentobarbitone (170 mg/kg)(CTS Chemical Industries, Tel Aviv, Israel). Both optic nerves were removed while still attached to the optic chiasma, and were immediately transferred to a vial containing a fresh salt solution consisting of 126 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2 mM $MgSO_4$, 2 mM $CaCl_2$ and 10 mM D-glucose, aerated with 95% O_2 and 5% CO_2 at room temperature. After 1 hour, electrophysiological recordings were made. In the injured nerve, recordings were made in a segment distal to the injury site. This segment contains axons of viable retinal ganglion cells that have escaped both primary and secondary damage, as well as the distal stumps of non-viable retinal ganglion cells that have not yet undergone Wallerian degeneration. The nerve ends were connected to two suction Ag-AgCl electrodes immersed in the bathing solution at 37 °C. A stimulating pulse was applied through the electrode, and the CAP was recorded by the distal electrode. A stimulator (SD9; Grass Medical Instruments, Quincy, Massachusetts) was used for supramaximal electrical stimulation at a rate of 1 Hz to ensure stimulation of all propagating axons in the nerve. The measured signal was transmitted to a microelectrode AC amplifier (model 1800; A-M Systems, Everett, Washington). The data were processed using the LabView 2.1.1 data acquisition and management system (National Instruments, Austin, Texas). For each nerve, the difference between the peak amplitude and the mean plateau of eight CAPs was computed and was considered as proportional to the number of propagating axons in the optic nerve. The experiments were done by experimenters 'blinded' to sample identity. In each experiment the data were normalized relative to the mean CAP of the uninjured nerves from PBS-injected rats.

Clinical evaluation of EAE. Animals were scored every 1–2 days according to the following neurological scale: 0, no abnormality; 1, tail atony; 2, hind limb paralysis; 3, paralysis extending to thoracic spine; 4, front limb paralysis; 5, moribund state.

Acknowledgments

We thank S. Smith and P. Taylor for editorial assistance, and I. Friedmann for help with graphics. I.R. Cohen is the incumbent of the Mauerberger Chair in Immunology, the director of the Robert Koch-Minerva Center for Research in Autoimmune Disease and the director of the Center for the Study of Emerging Diseases. M.S. holds the Maurice and Ilse Katz Professorial Chair in Neuroimmunology.

RECEIVED 24 AUGUST; ACCEPTED 3 NOVEMBER 1998

1. Streilein, J.W. Immune privilege as the result of local tissue barriers and immunosuppressive microenvironments. *Curr. Opin. Immunol.* **5**, 428–432 (1993).
2. Streilein, J.W. Unraveling immune privilege. *Science* **270**, 1158–1159 (1995).
3. Lazarov Spiegler, O. *et al.* Transplantation of activated macrophages overcomes central nervous system regrowth failure. *FASEB J.* **10**, 1296–1302 (1996).
4. Rapalino, O. *et al.* Implantation of stimulated homologous macrophages results in partial recovery of paraplegic rats. *Nature Med.* **4**, 814–821 (1998).
5. Hickey, W.F., Hsu, B.L. & Kimura, H. T-lymphocyte entry into the central nervous system. *J. Neurosci. Res.* **28**, 254–260 (1991).
6. Ben Nun, A., Wekerle, H. & Cohen, I.R. The rapid isolation of clonable antigen-specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. *Eur. J. Immunol.* **11**, 195–199 (1981).
7. Ota, K. *et al.* T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature* **346**, 183–187 (1990).
8. Martin, R. Immunological aspects of experimental allergic encephalomyelitis and multiple sclerosis and their application for new therapeutic strategies. *J. Neural Transm. Suppl.* **49**, 53–67 (1997).
9. Burns, J., Rosenzweig, A., Zweiman, B. & Lisak, R.P. Isolation of myelin basic protein-reactive T-cell lines from normal human blood. *Cell. Immunol.* **81**, 435–440 (1983).
10. Pette, M. *et al.* Myelin autoreactivity in multiple sclerosis: recognition of myelin basic protein in the context of HLA-DR2 products by T lymphocytes of multiple-sclerosis patients and healthy donors. *Proc. Natl. Acad. Sci. USA* **87**, 7968–7972 (1990).
11. Martin, R. *et al.* Fine specificity and HLA restriction of myelin basic protein-specific cytotoxic T cell lines from multiple sclerosis patients and healthy individuals. *J. Immunol.* **145**, 540–548 (1990).
12. Schluesener, H.J. & Wekerle, H. Autoaggressive T lymphocyte lines recognizing the encephalitogenic region of myelin basic protein: in vitro selection from unprimed rat T lymphocyte populations. *J. Immunol.* **135**, 3128–3133 (1985).
13. Hirschberg, D.L. *et al.* Accumulation of passively transferred primed T cells independently of their antigen specificity following central nervous system trauma. *J. Neuroimmunol.* **89**, 88–96 (1998).
14. Faden, A.I. & Salzman, S. Pharmacological strategies in CNS trauma. *Trends Pharmacol. Sci.* **13**, 29–35 (1992).
15. Faden, A.I. Experimental neurobiology of central nervous system trauma. *Crit. Rev. Neurobiol.* **7**, 175-186 (1993).
16. McIntosh, T.K. Novel pharmacologic therapies in the treatment of experimental traumatic brain injury: a review. *J. Neurotrauma* **10**, 215–261 (1993).
17. Lynch, D.R. & Dawson, T.M. Secondary mechanisms in neuronal trauma. *Curr. Opin. Neurol.* **7**, 510–516 (1994).
18. Bazan, N.G., Rodriguez de Turco, E.B. & Allan, G. Mediators of injury in neurotrauma: intracellular signal transduction and gene expression. *J. Neurotrauma* **12**, 791–814 (1995).
19. Liu, D., Yang, R., Yan, X. & McAdoo, D.J. Hydroxyl radicals generated in vivo kill neurons in the rat spinal cord: electrophysiological, histological, and neurochemical results. *J. Neurochem.* **62**, 37–44 (1994).
20. Yoshino, A., Hovda, D.A., Kawamata, T., Katayama, Y. & Becker, D.P. Dynamic changes in local cerebral glucose utilization following cerebral concussion in rats: evidence of a hyper- and subsequent hypometabolic state. *Brain Res.* **561**, 106–119 (1991).
21. Hovda, D.A., Yoshino, A., Kawamata, T., Katayama, Y. & Becker, D.P. Diffuse prolonged depression of cerebral oxidative metabolism following concussive brain injury in the rat: a cytochrome oxidase histochemistry study. *Brain Res.* **567**, 1–10 (1991).
22. Zivin, J.A. & Choi, D.W. Stroke therapy. *Sci. Am.* **265**, 56–63 (1991).
23. Yoles, E. *et al.* GM1 reduces injury-induced metabolic deficits and degeneration in the rat optic nerve. *Invest. Ophthalmol. Vis. Sci.* **33**, 3586–3591 (1992).
24. Yoles, E. & Schwartz, M. N-methyl-D-aspartate-receptor antagonist protects neurons from secondary degeneration after partial optic nerve crush. *J. Neurotrauma* **14**, 665–675 (1997).
25. Yoles, E. & Schwartz, M. Degeneration of spared axons following partial white matter lesion: Implications for optic nerve neuropathies. *Exp. Neurol.* **153**, 1–7 (1998).
26. Naparstek, Y. *et al.* T lymphocyte lines producing or vaccinating against autoimmune encephalomyelitis (EAE). Functional activation induces peanut agglutinin receptors and accumulation in the brain and thymus of line cells. *Eur. J. Immunol.* **13**, 418–423 (1983).
27. Popovich, P.G., Stokes, B.T. & Whitacre, C.C. Concept of autoimmunity following spinal cord injury: possible roles for T lymphocytes in the traumatized central nervous system. *J. Neurosci. Res.* **45**, 349–363 (1996).
28. Mor, F. & Cohen, I.R. Pathogenicity of T cells responsive to diverse cryptic epitopes of myelin basic protein in the Lewis rat. *J. Immunol.* **155**, 3693–3699 (1995).
29. Savio, T. & Schwab, M.E. Rat CNS white matter, but not gray matter, is nonpermissive for neuronal cell adhesion and fiber outgrowth. *J. Neurosci.* **9**, 1126–1133 (1989).
30. Lotan, M. & Schwartz, M. Cross talk between the immune system and the nervous system in response to injury: implications for regeneration. *FASEB J.* **8**, 1026–1033 (1994).
31. Zamvil, S.S. & Steinman, L. The T lymphocyte in experimental allergic encephalomyelitis. *Annu. Rev. Immunol.* **8**, 579–621 (1990).
32. Palladini, G., Grossi, M., Maleci, A., Lauro, G.M. & Guidetti, B. Immunocomplexes in rat and rabbit spinal cord after injury. *Exp. Neurol.* **95**, 639–651 (1987).
33. Mizrachi, Y. *et al.* Systemic humoral factors participating in the course of spinal cord injury. *Paraplegia* **21**, 287–293 (1983).
34. Cohen, I.R. Autoimmunity to chaperonins in the pathogenesis of arthritis and diabetes. *Annu. Rev. Immunol.* **9**, 567–589 (1991).
35. Mor, F. & Cohen, I.R. T cells in the lesion of experimental autoimmune encephalomyelitis. Enrichment for reactivities to myelin basic protein and to heat shock proteins. *J. Clin. Invest.* **90**, 2447-2455 (1992).
36. Ransom, B.R., Stys, P.K. & Waxman, S.G. The pathophysiology of anoxic injury in central nervous system white matter. *Stroke* **21**,11 (suppl), III52–57 (1990).
37. Barone, F.C., Feuerstein, G.Z. & White, R.F. Brain cooling during transient focal ischemia provides complete neuroprotection. *Neurosci. Biobehav. Rev.* **21**, 31–44 (1997).
38. Yarom, Y. *et al.* Immunospecific inhibition of nerve conduction by T lymphocytes reactive to basic protein of myelin. *Nature* **303**, 246–247 (1983).
39. Koller, H., Siebler, M. & Hartung, H.P. Immunologically induced electrophysiological dysfunction: implications for inflammatory diseases of the CNS and PNS. *Prog. Neurobiol.* **52**, 1–26 (1997).
40. Meeson, A.P., Piddlesden, S., Morgan, B.P. & Reynolds, R. The distribution of inflammatory demyelinated lesions in the central nervous system of rats with antibody-augmented demyelinating experimental allergic encephalomyelitis. *Exp. Neurol.* **129**, 299–310 (1994).
41. Ehrhard, P.B., Erb, P., Graumann, U. & Otten, U. Expression of nerve growth factor and nerve growth factor receptor tyrosine kinase Trk in activated CD4-positive T-cell clones. *Proc. Natl. Acad. Sci. USA* **90**, 10984–10988 (1993).
42. Lambiase, A. *et al.* Human CD4+ T cell clones produce and release nerve growth factor and express high-affinity nerve growth factor receptors. *J. Allergy Clin. Immunol.* **100**, 408–414 (1997).
43. Santambrogio, L. *et al.* Nerve growth factor production by lymphocytes. *J. Immunol.* **153**, 4488–4495 (1994).
44. Cohen, I.R. The cognitive paradigm and the immunological homunculus. *Immunol. Today* **13**, 490–494 (1992).
45. Lider, O., Reshef, T., Beraud, E., Ben Nun, A. & Cohen, I.R. Anti-idiotypic network induced by T cell vaccination against experimental autoimmune encephalomyelitis. *Science* **239**, 181–183 (1988).
46. Matzinger, P. Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* **12**, 991–1045 (1994).
47. Hirshfeld, H., Teitelbaum, D., Arnon, R. & Sela, M. Basic encephalitogenic protein: a simplified purification on sulfoethyl-Sephadex. *FEBS Lett.* **7**, 317 (1970).
48. Elias, D. *et al.* Vaccination against autoimmune mouse diabetes with a T-cell epitope of the human 65-kDa heat shock protein. *Proc. Natl. Acad. Sci. USA* **88**, 3088–3091 (1991).
49. Mor, F. & Cohen, I.R. Shifts in the epitopes of myelin basic protein recognized by Lewis rat T cells before, during, and after the induction of experimental autoimmune encephalomyelitis. *J. Clin. Invest.* **92**, 2199–2206 (1993).
50. Gillis, S., Ferm, M.M., Ou, W. & Smith, K.A. T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* **120**, 2027–2032 (1978).
51. Duvdevani, R. *et al.* Graded crush of the rat optic nerve as a brain injury model: combining electrophysiological, histological and behavioral outcome. *Restor. Neurol. Neurosci.* **2**, 31–38 (1990).
52. Hunig, T., Wallny, H.J., Hartley, J.K., Lawetzky, A. & Tiefenthaler, G. A monoclonal antibody to a constant determinant of the rat T cell antigen receptor that induces T cell activation. Differential reactivity with subsets of immature and mature T lymphocytes. *J. Exp. Med.* **169**, 73–86 (1989).
53. Yoles, E., Belkin, M. & Schwartz, M. HU-211, a nonpsychotropic cannabinoid, produces short- and long-term neuroprotection after optic nerve axotomy. *J. Neurotrauma* **13**, 49–57 (1996).