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Neuroprotection by T-cells depends on their subtype and activation state

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Abstract

This study analyzes how the antigen specificity, the subtype, and the activation state of T cells modulate their recently discovered neuroprotective potential. We assessed the prevention from neuronal damage in organotypic entorhinal–hippocampal slice cultures after co-culture with Th1 and Th2 cells either specific for myelin basic protein (MBP) or ovalbumin (OVA). We found that MBP-specific Th2 cells were the most effective in preventing central nervous system (CNS) tissue from secondary injury. This neuroprotective T cell effect appears to be mediated by soluble factors. After stimulation with phorbol myristate acetate and ionomycin, all T cells were most effective in preventing neuronal death. Our data show that the T cell subtype and activation state are important features in determining the neuroprotective potential of these cells.

Keywords: Neuroprotection; T cells; Organotypic slice culture; Hippocampus; MBP; Brain trauma

1. Introduction

Mechanical injury of the central nervous system (CNS) of mammalians results in primary damage of directly affected neurons and is often accompanied by a graduate, secondary loss of non-injured neurons (Faden and Salzman, 1992; McIntosh, 1993; Yoles and Schwartz, 1998a). Primary lesion causes changes in the extracellular ion concentration, enhances local inflammation (Liu et al., 1994), and increases the circulation of free radicals, the secretion of neurotransmitters, and the depletion of growth factors. These changes trigger a cascade of intracellular, destructive events leading to secondary neuronal death (Villegas-Perez et al., 1993; Berkelaar et al., 1994; Gracia-Valenzuela et al., 1994). In different rodent models of CNS-trauma, it has been shown that CNS-specific autoreactive T cells were capable of protecting nervous tissue from this secondary injury (review: Cohen and Schwartz, 2000). Autoimmune T cells specific for myelin basic protein (MBP) could prevent neurons from the spread of neuronal death caused by primary injury to the spinal cord (Moalem et al., 1999; Hauben et al., 2000a). T cells specific for Copaxone 1, a compound used for multiple sclerosis therapy, could decrease secondary degeneration after optic nerve crush (Kipnis et al., 2000). The passive and active immunization with myelin peptides (MBP, PLP, MOG) is protective against neuronal death in spinal cord injury (Hauben et al., 2000b) and optic nerve crush (Fisher et al., 2001). In addition, it has been shown that MBP-specific T cells could prevent cyst formation in the spinal cord injury model (Butovsky et al., 2001). Different authors have reported an accumulation of T cells at the site of degeneration and secondary regeneration (Hirschberg and Schwartz, 1995; Raivich et al., 1998; Moalem et al., 1999; Butovsky et al., 2001; Bechmann et al., 2001).

Past studies on protective immune effects used either undefined or autoreactive T cells of the Th1 subtype. All of these studies (except those using Copaxone 1 specific T cells) reported a transient autoimmune response as a side effect after application of CNS-specific T cells. It is supposed that autoreactive CNS-specific Th1 cells trigger autoimmune diseases like multiple sclerosis (MS) and its rodent model, experimental autoimmune encephalomyelitis (EAE) (Ben-Nun and Cohen, 1982; Zamvil and Steinman, 1990). Except in severely immunodeficient RAG-1 knockout mice

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where they actively induced an atypical form of EAE (Lafaille et al., 1997), myelin specific Th2 cells do not induce EAE in normal animals (Khoruts et al., 1995; Lafaille et al., 1997) and support anti-inflammatory properties of the brain (Gimsa et al., 2001). Therefore, in the present study, we investigated whether Th1 and Th2 cells differ in their potential to prevent secondary degeneration after mechanical CNS-damage. We were specifically interested in finding out the extent to which the antigen specificity and the activation state of Th1 and Th2 cells are able to modulate their neuroprotective potential.

2. Materials and methods

2.1. Organotypic slice cultures (OSC)

OSCs were prepared from either 11-day-old B10.PL mice (Jackson Laboratories, Boston, MA, USA) or 11-day-old BALB/c mice (Charles River, Sulzfeld, Germany) as previously described (Dieckmann et al., 1994; Hailer et al., 1996; Kluge et al., 1998). After decapitation, the brains were rapidly removed under sterile conditions and placed in ice-cold preparation medium consisting of minimum essential medium (MEM, Gibco) with 1% L-glutamine (Gibco) at pH 7.35. The hippocampi were taken from the brain and cut into 350 μm thick vertical slices on a tissue chopper (Technical Products International, St. Louis, MO, USA). The slices were cultured on Millipore cell culture inserts, pore size 0.4 μm (Millipore, USA), in six-well plates containing cultivation medium. The sterile cultivation medium contained 50% MEM, 25% HBSS (Gibco), 25% heat-inactivated normal horse serum (Gibco), 2% glutamine, 10 μg/ml insulin–transferrin–sodium selenite supplement (Boehringer Mannheim, Germany), 2.64 mg/ml glucose (Braun, Melsungen, Germany), 0.1 mg/ml streptomycin (Sigma), 100 U/ml penicillin (Sigma), and 0.8 μg/ml vitamin C (Sigma) at pH 7.35. The OSCs were incubated at 35 °C in a humidified atmosphere with 5% CO_2.

2.2. Th1 and Th2 cell lines

To prepare Th1 and Th2 cell lines, we used either transgenic mice carrying a TCR for MBP peptide Ac1-11 (the kind gift from David Wraith, Bristol) (Lui et al., 1995) or transgenic mice carrying a TCR DO11.10 for $cOVA_{323-339}$ peptide (the kind gift from Monika Brunner-Weinzierl, DRFZ Berlin). In short, we cultured mixed splenic and lymph node cell cultures in RPMI medium (Gibco) supplemented with 10% FCS, 0.1 mg/ml streptomycin (Sigma), 100 U/ml penicillin (Sigma) and 0.01% mercaptoethanol with either 3 μg/ml MBP-peptide Ac1-11 (Pepscan Immunoanalytic, Berlin, Germany) or 1 μg/ml $cOVA_{323-339}$ peptide (Affina, Netherlands) in a total volume of 5 ml in six-well plates. This was done in the presence of either 1 μg/ml recombinant mouse IL-12 (R&D Systems, Abingdon, UK) and 1 μg/ml anti-mouse IL-4 (Pharmingen), or 1 mg/ml recombinant mouse IL-4 (Biosource, Europe) and 1 μg/ml anti-mouse IL-12 (R&D Systems) to obtain Th1 or Th2 cell lines, respectively. After 3–4 days in vitro, the T cells were diluted 1:2 and supplemented with IL-2 (100 U/ml) to ensure optimal proliferation. After 7 days in vitro, T cells were specifically restimulated with their antigen by irradiated splenocytes functioning as APCs. The cell lines obtained exhibited a clear Th1 or Th2 profile, as detected by two-side sandwich ELISAs for IFN-γ and IL-4 (Pharmingen). After restimulation, the bulk of the cells was cultured for 48 h, and CD4+ T cells were isolated by high gradient magnetic cell sorting (anti-CD4 MACS beads; Miltenyi Biotec; Bergisch-Gladbach, Germany). FACS analysis showed approx. 95% CD4+ cells. T cells were washed twice in OSC cultivation medium. Immediately after preparation, the T cells were added to the cultivation medium of acute slices beneath the culture membrane at a concentration of approx. 10^6/ml supplemented with irradiated splenocytes as APCs at a 1:2 ratio either with or without antigen. B10.PL slices were co-cultured with MBP-specific T cells, and BALB/c slices were co-cultured with ovalbumin (OVA)-specific T cells. To perform non-specific activation, MBP- and OVA-specific Th1 and Th2 cells were incubated with phorbolmyristate esther (SIGMA, 5 ng/ml) and ionomycin (Sigma, 1 μg/ml) in T cell cultivation medium for 5 h and washed twice in OSC cultivation medium before co-culture.

2.3. Propidiumiodide staining

To stain the nuclei of dead cells, the slices were incubated 24 h after co-culture with propidiumiodide (PI; 1 μg/ml) in OSC cultivation medium for 30 min followed by three washing steps with OSC cultivation medium for 10 min each.

Slice cultures were fixed with 4% paraformaldehyde in PBS for 4 h at 4 °C and washed twice with PBS. Fixed slices were incubated in phosphate buffered sucrose solution at 0.8 M to 1.4 M for 1 week before horizontal sectioning on a Jung cryostat 2800 Frigocut-E (Cambridge Instruments, Nussloch, Germany) at −31 °C. Slices were cut into 20 μm sections for fluorescence microscopy. The excitation wavelength of PI lies between 740 and 760 nm. Therefore, a green fluorescence filter was used to observe the staining.

2.4. Picture analysis and quantification

In two separate experiments, all of the 12 experimental groups and 12 matching control groups contained five to eight slices. Each slice had been cut into 12 to 15 sections (20 μm) and pictures were taken of the sections in the virtual depth of approximately 60 μm. The analysis of the resulting 336 pictures containing the granular cell layer was obtained with a specialized computer program based on AMBA software. The read out was defined as dead cells per 100,000 μm^2, considering cells with a PI stained nucleus

to be dead. Each experimental group had been normalized to its control, and the data were presented using the controls as a baseline. All bar graphs show the number of neurons per 100,000 μm^2 prevented from secondary degeneration by treatment with T cells compared to the control slices. The statistical analysis was performed using a Friedman-test for differences between subtypes (Th1; Th2; K) in the groups ± antigen or ± contact (MBP only). The effect of antigen was tested pairwise (± antigen) in each group (Th1; Th2; K) with a Mann–Whitney *U*-test. To test the protective potential of MBP- and OVA-specific Th1 and Th2 cells treated with PMA + Ionomycin (5 ng/ml/1 μg/ml), a one-way-ANOVA-test followed by four pairwise contrast *t*-tests (experimental group/control) was performed. The bars in the diagrams (Figs. 1–3,5–7) represent the means of the data per group. The standard deviation (SD) for each mean is shown as a scale above each bar. Significant differences are marked with an asterisk.

2.5. Neurofilament staining

To confirm that the nuclei of dead cells belong to neurons, a staining of the sectioned slices was carried out using rabbit-anti-neurofilament 68 kDa (Chemicon International, CA, USA; 1:80) as a primary antibody and anti-rabbit-ALEXA 488 (Molecular Probes, USA; 1:200) as secondary antibody.

2.6. Cytokine ELISAs

IFN-γ and IL-4 levels in supernatants of T cells and slices were determined by ELISAs (OptEIA™ ELISA sets, Pharmingen, sensitivities: 31.3 pg/ml, and 7.8 pg/ml for IFN-γ and IL-4, respectively) following the manufacturer's instructions. Supernatants from T cells were taken 48 h after the first restimulation; supernatants from the slices were taken before fixation.

2.7. Flow cytometry

Samples for flow cytometry were taken 48 h after restimulation (start of the co-culture with the slices), fixed in 2% paraformaldehyde in PBS for 15 min at room temperature and kept refrigerated until staining for flow cytometry. This procedure allowed us to perform FACS analysis for subsequent experiments and cell types at the same time point. Cells were double-stained for CD4 and one of the surface molecules CD25, CD44, CD49d, CD69, CD71 and CD154 (1 μg/ml; clones PC61, IM7, R1-2, H1.2F3, C2, and MR1, respectively, from Pharmingen) in PBS/0.5% BSA/0.01% NaN_3 for 10 min at room temperature. After washing with PBS, staining was analyzed on a FACStrak™ flow cytometer (Becton Dickinson, Mountainview, USA). Samples were gated on live lymphocytes based on forward and side scatter parameters (10,000 events/sample) and analyzed using CellQuest software (Becton Dickinson). Statistical analysis was performed using a Friedman-test for each activation marker.

3. Results

3.1. Activation state and cytokine production of MBP- and OVA-specific Th1 and Th2 cells

To observe whether MBP- and OVA-specific Th1 and Th2 cells were equally activated, culture samples were collected 48 h after their first restimulation, then fixed and analyzed for the expression of the T cell activation markers CD25, CD44, CD49d, CD69, CD71 and CD154 (CD40L) by flow cytometry. CD25 is the α-chain of the IL-2 receptor that is only expressed by activated T cells to form the high affinity IL-2 receptor, which enables T cell proliferation. CD44, the leukocyte common antigen, is one of the migration-enabling adhesion molecules of T cells. CD49d, the α-chain of VLA-4, enables activated T cells to cross the blood–brain barrier and induces TNF-α production after ligation with VCAM-1 on microglia (Chabot et al., 1997). CD69 is the "very early activation antigen". Proliferating cells express the transferrin receptor CD71. CD154 (CD40L) binds to CD40 on antigen-presenting cells and may induce IL-12 production by microglia (Aloisi et al., 1999) and upregulate the costimulatory molecules B7-1 and B7-2 (Grewal and Flavell, 1996). Flow cytometry showed that antigen-specific restimulated MBP- and OVA-specific Th1 and Th2 cells were equally activated when added to the slice cultures (Fig. 1). CD25, CD49d, CD69,0 and CD71 were expressed two- to four-fold higher in PMA + Ionomycin-treated cells than in antigen-specific restimulated T cells ($p \leq 0.05$). Again, there were no differences observed in the

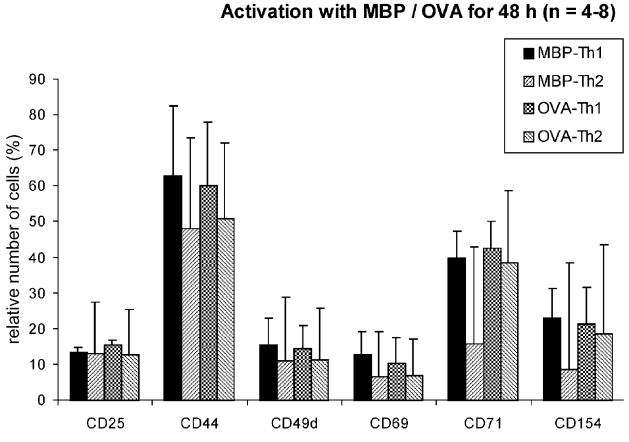

Fig. 1. State of activation of MBP- and OVA-specific Th1 and Th2 cells 48 h after antigen specific stimulation. The expression of the activation markers was observed in eight (CD25, CD44, CD49, CD69 and CD71) and four (CD154) independent experiments for MBP- and five independent experiments for OVA-specific T cells of each cell subtype. The number of cells positive for an activation marker was set in relation to the total number of cells. There was no significant difference in the activation state between Th1 and Th2 cells.

activation state between MBP- or OVA-specific Th1 and Th2 cells after the same treatment (Fig. 2).

The commitment of T cells to the Th1 and Th2 phenotype was ensured by ELISA. The samples were taken 48 h after restimulation from the supernatant of approximately 10^7 Th1 and Th2 cells (2×10^6 cells/ml), respectively. We found high IFN-γ but no IL-4 secretion by Th1 cells, and high IL-4 but no IFN-γ secretion by Th2 cells, as expected (Fig. 3). Both Th1 and Th2 continued to secrete either IFN-γ or IL-4 when co-cultured with slices (10^6 cells/ml; four slices shared 1 ml supernatant). No difference was observed in the cytokine profile between the T cells activated with either PMA + Ionomycin or antigen and APCs 48 h after stimulation, although the amount of IFN-γ and IL-4 increased when cells were stimulated with PMA + Ionomycin.

3.2. Protective T cell effects are contact independent

Both subtypes of MBP-specific T cells were able to protect the neurons from secondary degeneration (Fig. 4a–e). In Fig. 4f, a representative photomicrograph shows a double labeling with neurofilament and propidiumiodide to prove that almost all of the dead cells are neurons. The co-culture of slices with either Th1 or Th2 lacking contact between T cells and slices resulted in a significant reduction of neuronal damage ($p \leq 0.05$). Th2 cells were twice as effective as Th1 cells ($p \leq 0.01$). The neuronal survival rate was three-fold lower when contact between Th1 or Th2 with brain tissue was allowed ($p \leq 0.02$). The MBP-specific T cells were much more protective without contact to the slices (Fig. 5). Therefore, all following experiments were performed without contact between T cells and brain tissue.

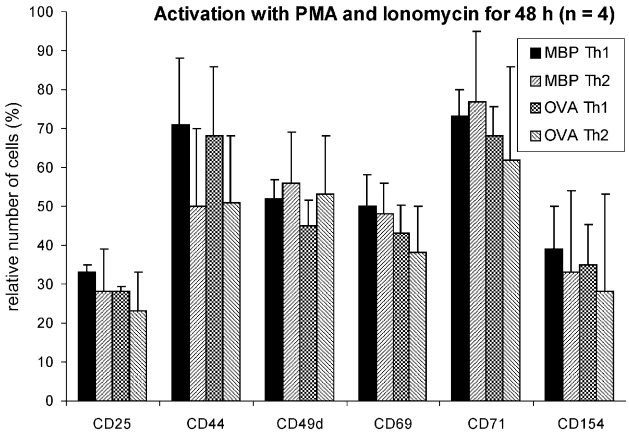

Fig. 2. Activation state 48 h after cultivation of MBP- and OVA-specific Th1 and Th2 cells following 5 h of stimulation with PMA and ionomycin (5 ng/ml/1 μg/ml). The expression of the activation markers was observed in five independent experiments for each cell subtype. The number of cells positive for an activation marker was set in relation to the total number of cells. There was no significant difference in the activation state between the subtypes of the T cells. CD25, CD49d, CD69 and CD71 were expressed two- to four-fold higher in PMA + Ionomycin-treated cells than in antigen specific-restimulated T cells (Friedman-tests, $p \leq 0.05$).

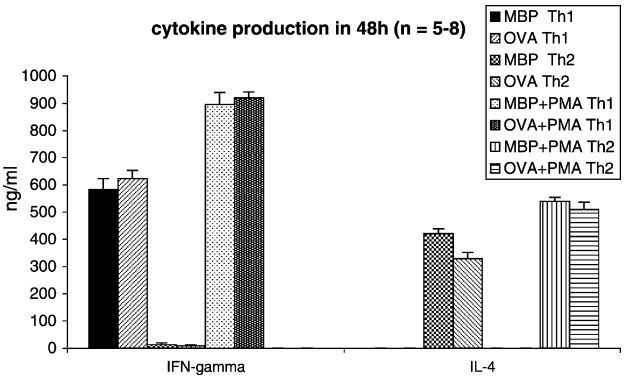

Fig. 3. Cytokine production of MBP- and OVA-specific Th1 and Th2 cells (3–5 million cells) 48 h after restimulation with either antigen or PMA + Ionomycin. The cytokine profile of the T cells was checked with ELISA. High IFN-γ but no IL-4 secretion by MBP- and OVA-specific Th1 cells, and high IL-4 but very low IFN-γ secretion by MBP- and OVA-specific Th2 cells was observed as expected. The cytokine production of PMA + Ionomycin stimulated T cells was higher than of those T cells stimulated with antigen. This is most likely due to the higher state of activation. The samples were taken from the supernatants of 3–5 million cells from five independent experiments with OVA T cells and eight independent experiments with MBP specific T cells prior to co-culture with the slices.

3.3. MBP-specific Th2 cells are the most potent T cells in protecting neurons

To investigate the effect of CNS-specificity and the influence of antigen-specific stimulation, we performed additional experiments with OVA-specific Th1 and Th2 cells (Fig. 6). Only the OVA-specific Th1 cells were unable to protect at all. All other cells had the potential to prevent the slices from secondary neuronal degeneration, but to different degrees ($p \leq 0.01$). The most potent cells were the MBP-specific Th2 cells. Without the addition of antigen, the neuronal survival in their presence was three-fold higher than in the presence of OVA-specific Th2 cells. There was no longer a significant difference when antigen was added to the co-culture. Here, both MBP- and OVA-specific Th2 cells were neuroprotective. The difference between MBP-specific Th1 and Th2 cells persisted in this set of experiments ($p \leq 0.03$). The addition of antigen did not significantly alter the neuroprotective effect of MBP-specific T cells. Considering only the OVA-specific Th2 cells, a tendency towards a higher protective potential could be described when antigen was added. OVA-specific Th1 cells showed an increased protective potential when antigen was added ($p \leq 0.05$).

3.4. High activation state of T cells is crucial for neuroprotection

To exclude an influence of splenocytes used as APCs for T cell stimulation in the co-culture and to perform an adequate activation at the same time, we incubated pure

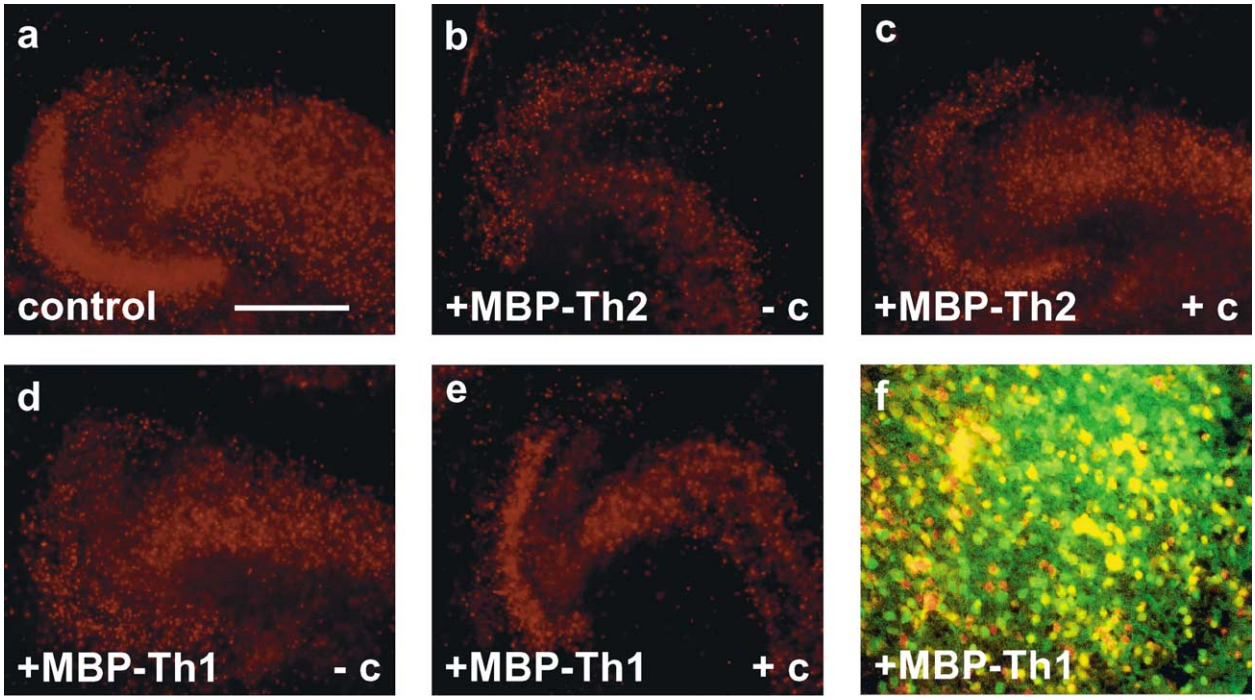

Fig. 4. Representative photomicrographs show neuronal damage in slices 24 h after treatment with MBP-specific Th1 and Th2 cells. The nuclei of dead cells are stained with propidiumiodide and appear in a red fluorescence. (a) Control slices without T cells showed the most neuronal damage. (b) MBP-specific Th2 cells without contact (– c) showed the highest protective potential. (c) When MBP-specific Th2 cells were in contact (+ c) with the brain tissue, their protective potential was significantly decreased. (d) MBP-specific Th1 cells without contact to the slices were also neuroprotective. (e) MBP-specific Th1 cells in contact with neuronal tissue were not protective. (f) Double staining of neurons with neurofilament and propidiumiodide shows that the nuclei of dead cells belong to neurons in this region of the hippocampus. This micrograph shows the survival rate after treatment with MBP-specific Th1 cells without contact to the slices. Scale bar in picture (a) is representative for all photomicrographs: 0.5 μm.

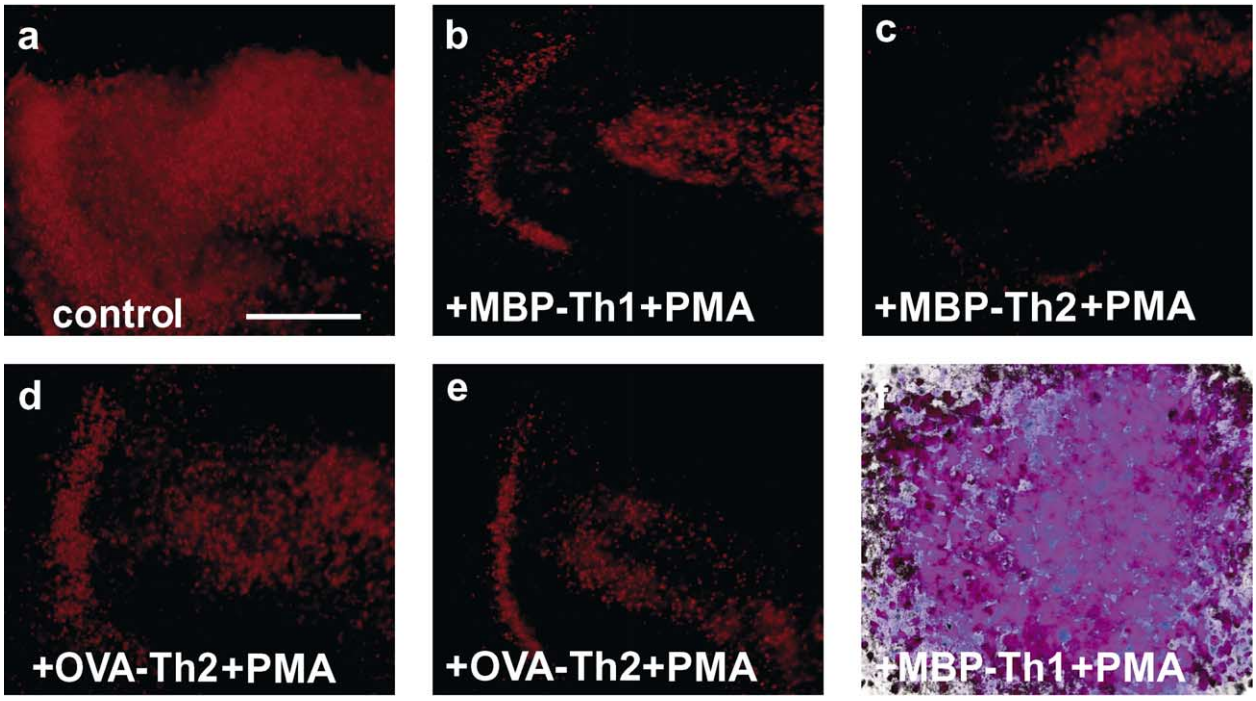

Fig. 8. Representative photomicrographs show the neuronal damage in slices depicted by propidiumiodide staining of the nuclei of dead cells. (a) Control slices represent the maximal neuronal damage, which resemble the baseline in the diagrams. (b–e) All T cell subsets were neuroprotective when pre-treated with PMA + Ionomycin (5 ng/ml/1 μg/ml) for 5 h and cultivated 48 h prior to co-culture. No difference was observed between Th1 and Th2 or OVA- and MBP-specific T cells. (f) This photomicrograph shows a representative picture of double-labelled neurons with neurofilament (green) and propidiumiodide (red) after treatment with PMA + Ionomycin activated MBP-specific Th1 cells. Almost all neurons were alive as shown by the low numbers of yellow stained (double-labelled) cells. The scale bar in (a) is representative for all photomicrographs: 0.5 mm.

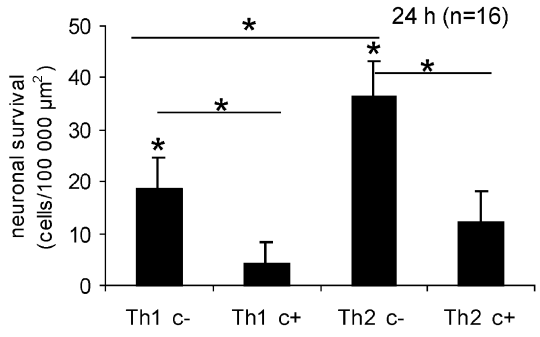

Fig. 5. Neuronal survival in slices 24 h after treatment with MBP-specific Th1 and Th2 cells with (+c) and without (−c) cell–cell contact. Two independent experiments with eight slices per group were performed. The baseline represents the neuronal survival in the controls. The bars show the additional numbers of surviving neurons after treatment with cells. An asterisk directly placed over a bar shows the significant difference between control and treatment group ($*p \leq 0.05$). An asterisk placed over a line shows the significant differences between Th1 and Th2 within the group without cell–cell contact (−c) and the difference between +/− contact in the groups Th1 and Th2. MBP-specific Th2 cells without contact to the brain tissue could prevent most of the neurons from death. Their protective potential was three-fold higher than that of MBP-specific Th2 cells with contact to the slices ($*p \leq 0.02$) and two-fold higher than this of MBP-specific Th1 cells without contact to brain tissue ($*p = 0.01$). The survival rate of neurons after treatment with MBP-specific Th1 cells without cell–cell contact was three-fold higher than that of the same cells when contact to the slice was allowed ($*p \leq 0.02$). MBP-specific Th1 cells in contact with brain tissue were not protective at all.

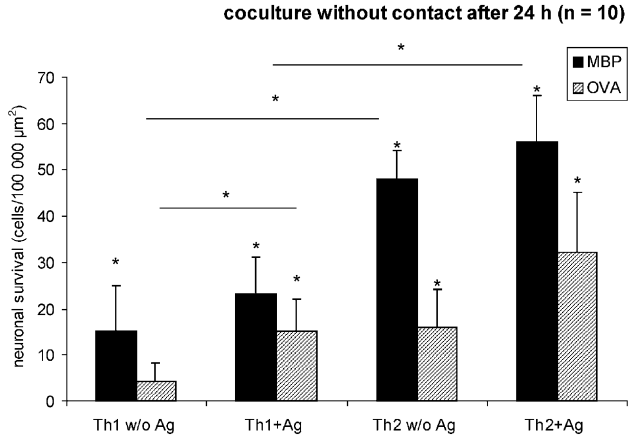

Fig. 6. Neuronal survival in slices 24 h after treatment with MBP- and OVA-specific Th1 and Th2 cells with (+Ag) and without antigen (−Ag) without contact to the brain tissue. Two independent experiments with five slices in each experimental group had been performed. The baseline represents the neuronal survival in the controls. The bars show the additional numbers of surviving neurons after treatment with cells. An asterisk placed directly over the bars shows the significant difference between the treatments and the controls. All T cells, except OVA-specific Th1 cells without antigen, were neuroprotective ($*p \leq 0.01$). An asterisk placed over a line shows the significant difference between the treatments with Th1 and Th2 cells. MBP-specific Th2 cells without antigen exhibited a two- to three-fold higher protective potential than MBP-specific Th1 cells and OVA-specific Th2 cells without antigen ($*p \leq 0.03$). The addition of antigen did not alter the protective potential of the MBP-specific Th1 and Th2 cells. After addition of antigen OVA-specific Th1 showed an increased protection potential ($*p = 0.05$). A tendency of OVA-specific Th2 cells to increase the neuronal survival when activated with antigen could be observed.

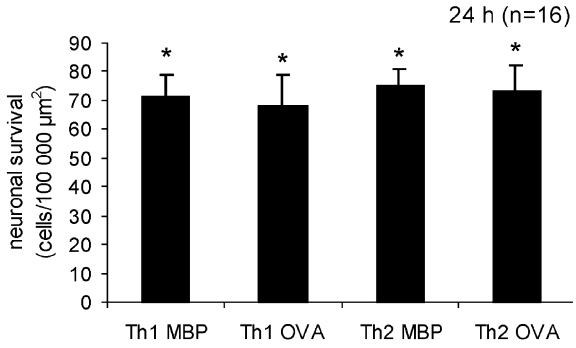

Fig. 7. Neuronal survival in slices 24 h after treatment with MBP- and OVA-specific Th1 and Th2 cells after PMA+Ionomycin stimulation without contact to the slices. All T cell subsets were neuroprotective in comparison to the controls ($*p \leq 0.005$). The baseline represents the survival rate in the controls. The bars show the additional number of surviving neurons per 100,000 µm². In two independent experiments, eight slices per group were analyzed.

MBP- and OVA-specific Th1 and Th2 cells with PMA+Ionomycin (5 h, 5 ng/ml/1 µg/ml) prior to co-cultivation. All T cells were able to protect the same high amount of neurons from secondary degeneration ($p \leq 0.005$). Under this circumstance, neither the subtype nor the CNS-specificity had an influence on the outcome (Figs. 7 and 8a–e). Although it should be kept in mind that PMA stimulation reflects a rather non-physiological situation, this indicates that presumably every T cell has a potential to produce soluble factors that could lead to a neuroprotective effect. In Fig. 8f, a representative photomicrograph shows a double labeling with neurofilament and propidiumiodid to prove that most of the dead cells are neurons.

4. Discussion

In this study using a cortical model, we could show the direct effect of T cells on neuronal survival in vitro. Our main findings are that: (I) the mediators of the neuronal survival are soluble and that the strength of the effect depends mostly on (II) the activation state and (III) the subtype of the T cells.

In the context of nerve trauma, the discovery that macrophages can facilitate CNS repair (Lazarov-Spiegler et al.,1996; Prewitt et al., 1997; Rabchevsky and Streit, 1997; Papalino et al., 1998) set the stage for reconsideration of autoimmunity as an exclusively destructive process (Schwartz et al., 1999a,b). It has been shown that autoreactive T cells that evoke autoimmune diseases like MS or EAE could be benign after CNS injury. Autoimmune T cells specific for MBP could prevent neurons from the spread of neuronal death caused by primary injury to the spinal cord (Moalem et al., 1999; Hauben et al., 2000a) and cyst formation (Butovsky et al., 2001). T cells specific for Copaxone 1, a synthetic peptide highly similar to parts of MBP, could decrease secondary degeneration after optic

nerve crush (Kipnis et al., 2000). The passive and active immunization with peptides of myelin (MBP, PLP, MOG) is protective against neuronal death in the case of spinal cord injury (Hauben et al., 2000b) and optic nerve crush (Fisher et al., 2001). A recent study using different experimental paradigms in rodents has shown that CNS trauma spontaneously evokes a beneficial T cell-dependent immune response that reduces neuronal cell loss (Yoles et al., 2001). In this study, we could refer to the potential of T cells to protect neurons from secondary damage following primary mechanical injury in the course of explantation from the living animal into the culture dish. In prior studies, this approach had already allowed us to analyze neuronal survival/damage following invasion of microglial cells (Heppner et al., 1998; Ullrich et al., 2001) and lymphocytes into the brain slice (Gimsa et al., 2000, 2001). Here, we took advantage of the same system to question the role of cell–cell interaction, soluble factors, the activation state and subtype, as well as antigen specificity of T cells on neuronal survival/damage.

According to our data, highly activated T cells could be protective against neuronal death independent of their subtype or antigen specificity. When stimulated with PMA and ionomycin (shown by FACS analysis of activation markers), the activation state of the T cells appeared to be more crucial than the subtype and antigen specificity. It has been reported that a combination of phorbol 12-myristate 13-acetate (PMA), protein kinase C activator and ionomycin induces CD4+ cells that express bcl-2, TCR, CD69, CD5 and HAS. Those surface markers resemble those on thymocytes challenged with antigen (Ohoka et al., 1996). PMA + Ionomycin-treated T cells showed resistance to glucocorticoid-induced apoptosis (Zhao et al., 1995) as well as to DEX-induced apoptosis (Tanahashi et al., 2001). This leads to the assumption that the PMA + Ionomycin-treated T cells in our study are not only highly activated, but also more viable than those treated with antigen. This could explain the increased neuroprotective potential of the PMA + Ionomycin-treated cells. However, stimulation with PMA and Ionomycin is rather non-physiological and differs from antigen-specific stimulation. Therefore, it is possible that the neuroprotective effects seen in both situations depend on different pathways. This opens up the possibility that other cell populations are also able to exert protective effects after unspecific stimulation.

A more physiological state that can be compared to the in vivo situation is achieved by our experiments using antigen-specific stimulation. OVA-specific T cells were more protective when antigen was added, whereas MBP-specific T cells showed no difference in their protective potential. This important difference indicates an antigen-specific effect where MBP-specific T cells find their antigen in the supernatants of the slices and become activated when co-cultured with irradiated splenocytes that presented the MBP antigen to them. Further, our data show that an antigen-specific activation could increase the protective potential of T cells, although not as much as stimulation with PMA and ionomycin.

Our findings support the idea that soluble factors released by T cells are the active agents resulting in neuroprotection. We could show that neuroprotection is achieved in the absence of contact between T cells and brain tissue, while invading T cells showed less neuroprotective effects. It is feasible to explain this important difference by a direct cell-to-cell damage brought about by T cells when they are allowed to invade into the slice, which probably overrules their protective potential mediated by soluble factors. Our experimental setting, using a two-compartment approach allowing molecules, but not cells, to shift from the brain slice compartment to the T cell compartment, enabled us to differentiate both (destructive versus beneficial) effects. Since MBP-specific Th2 cells exhibited the highest potential to protect neurons from secondary death in our system, Th2 cells probably produced these factors in a larger amount than Th1 cells. Besides anti-inflammatory cytokines like IL-10 or TGF-β, neurotrophic factors (NT) could be potential candidates for further investigations. It has been reported that NTs, such as BDNF, NGF, NT-3 and NT-4/5, are produced and continuously released at the site of injury by activated T cells (Erhard et al., 1993; Kerschensteiner et al., 1999; Flügel et al., 2001). Moalem et al. (2000) suggested that the neuroprotective effect of autoreactive T cells involves the secretion of factors, such as NTs, by the T cells reactivated by their specific antigen in the injured CNS. Cellular carriers like activated MBP-specific Th2 cells might result in a therapeutic application of NTs at the site of CNS injury.

It has been shown that CNS-specific T cells cross the blood–brain barrier (Hickey et al., 1991) and specifically accumulate at the site of CNS lesions (Hirschberg et al., 1998; Raivich et al., 1998). MBP-specific Th2 cells seem to be even more effective in migrating through an artificial blood–brain barrier than MBP-specific Th1 cells (Biernacki et al., 2001). It is also possible that the activated T cells in our study were capable of minimizing the effect of substances that are dangerous to neurons like glutamate or thrombin, which are released after injury in a non-physiological amount (Dreyer et al., 1996; Yoles and Schwartz, 1998b; Friedmann et al., 1999, 2001). It is of importance to note, however, that the protective effects analyzed in our study concern the survival of neurons, not their regenerative capacity. It has to be unveiled in further studies whether the same subset of T cells mediating neuronal survival also plays a role in mechanisms of axonal plasticity, which is essential for functional recovery after trauma. In fact, a critical role for Th1 cells in post-traumatic protective autoimmunity has been demonstrated (Kipnis et al., in press). This indicates that beneficial T cell effects should be considered a complex, concerted action by different T cell subtypes on the survival of neurons and regrowth of their axons following trauma.

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