Lymphocyte-mediated neuroprotection in *in vitro* models of excitotoxicity involves astrocytic activation and the inhibition of MAP kinase signalling pathways.

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Abbreviations: APCs, antigen presenting cells; BBB, blood-brain barrier; CM, conditioned media; CNS, Central nervous system, ERK, extracellular signal-regulated kinases; FAc, fluoroacetate; GFP, green fluorescent protein; KA, kainate; LCM, lymphocyte conditioned media; MAPK, mitogen activated protein kinases; MHC, major histocompatibility complex; OSCs, organotypic hippocampal slice cultures; OGD, oxygen-glucose deprivation;
Abstract

It is well established that immunosurveillance is active in the CNS and plays a key role in several CNS disorders but the exact role of immune cells remains elusive. Thus, in the present study we investigated whether lymphocytes are protective / detrimental in *in vitro* models of excitotoxicity. Kainate (KA)-induced neuronal death was significantly reduced following exposure to mixed lymphocytes or purified T lymphocytes containing either activated or non-activated T-lymphocytes. Conditioned media from lymphocyte preparations, but not boiled conditioned media, was protective against KA-induced toxicity indicating soluble mediators underlie the observed neuroprotection with cytokine arrays indicating IL-16 as the likely candidate. A role for astrocytes was established as the neuroprotection was abolished in the presence of the glial toxin, fluoroacetate. Furthermore, lymphocytes inhibited p38 MAPK and ERK signalling pathways with pharmacological inhibition of these pathways mimicking the protective effect of lymphocytes. Similarly, lymphocytes were neuroprotective against oxygen-glucose deprivation (OGD)-induced cell death with the inhibition of p38 MAPK and ERK signalling pathways involved. These data indicate that lymphocytes are neuroprotective under our experimental conditions and we suggest that astrocytic activation and inhibition of MAPK signalling cascades are involved but further studies are required to investigate whether similar mechanisms underlie the actions of lymphocytes in *in vivo* experimental models of disease.

**Keywords:** astrocytes, lymphocytes, MAP kinases, neuroinflammation, neuroprotection, T cells.
1. Introduction

Within the brain, there is an absence of lymphatic vessels and the classical major histocompatibility complex (MHC) molecules, leading to the suggestion that the brain shows limited immune surveillance or is an immunologically privileged area (Becher et al., 2006; Engelhardt, 2006). However, it is evident that the brain is not ‘immune-privileged’ as it is now well established that immune cells can cross the blood-brain barrier (BBB) (Hickey, 1999, Becher et al., 2006) and extensive studies have suggested that immune cells can play both detrimental or protective roles in central nervous system (CNS) function (for reviews see Cohen and Schwartz, 1999; Schwartz, 2005; Dittel, 2008).

It has been proposed that activated T cells cross the BBB irrespective of antigen specificity and, following recognition of specific antigens by antigen presenting cells (APCs) including astrocytes, dendritic cells and microglia, remain in the CNS to initiate an inflammatory reaction (Hickey et al., 1991; Liblau et al., 2013). Within the CNS, T cells have the capacity to proliferate, interact with resident APCs and produce cytokines that exacerbate the inflammatory response, which may contribute to the neurological symptoms observed in certain CNS diseases including amyotrophic lateral sclerosis and multiple sclerosis (Ponomarev et al., 2004, 2007; Batoulis et al., 2011). Indeed, aseptic cerebral injury is reduced in T cell-deficient recombinase activating gene knock out (RAG -/-) mice whereas cerebral injury is exacerbated when activated CD4+ T cells are re-introduced into these mice (Fee et al., 2003). In contrast to the general concept that T cell infiltration is detrimental to the CNS, T cells and B cells can also contribute to the maintenance of normal physiology (Cohen and Schwartz, 1999; Kipnis et al., 2004; Ziv et al., 2006; Wolf et al., 2009) and there is increasing evidence that T cells may be beneficial following CNS trauma (Cohen and Schwartz, 1999; Schwartz, 2005). An example of this is the impaired survival of facial motor
neurons in severe combined immunodeficient (SCID) mice after experimental facial nerve axotomy, which is restored following transfer of wild-type splenocytes (a mixture of T and B cells) (Serpe et al., 1999). Moreover, adoptive transfer of T cells reactive to Cop-1 following injury also increases the number of surviving neurons (Kipnis et al., 2000). The underlying mechanism(s) by which lymphocytes rescue neurons is not clear but it is proposed that T cells are capable of producing neurotrophic factors (Moalem et al., 2000) and inducing glutamate clearance by astrocytes (Garg et al., 2008) and microglia (Shaked et al., 2005) which may contribute to their neuroprotective role.

Whether lymphocytes are detrimental or protective to the CNS may depend on the pathological conditions under which these cells infiltrate the CNS. In the present study, we have utilised organotypic slice cultures (OSCs) to investigate how lymphocytes modulate cell death induced by either exposure to kainate (KA) or oxygen glucose deprivation (OGD). OSCs were utilised in the current study as they are used extensively as a model for investigating neurodegenerative diseases and to study mechanisms involved in neuroprotection (Wang et al., 2005; Cho et al., 2007) and importantly for the current study, OSCs maintain their inflammatory responses under our culture conditions (Huuskonen et al., 2005). We show that cell death induced by both toxic insults is reduced in the presence of lymphocytes and this protection is independent of the T cell activation state. Furthermore, we demonstrate that this neuroprotection involves astrocytic activation via soluble mediators, with IL-16 being the lead candidate, and the inhibition of p38 mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinases (ERK) signalling pathways. These data indicate that under the current experimental conditions, lymphocytes are neuroprotective and suggest therapeutic opportunities which may prove useful in future interventions in pathological CNS conditions.
2. Materials and Methods

2.1 Materials

(2S,3S,4S)-3- (carboxymethyl)-4-(prop-1-en-2-yl)pyrrolidine-2-carboxylic acid (Kainic acid, KA), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7- sulfonamide (NBQX), DL-(-)-2-amino-5-phosphonopentanoic acid (DL-AP5), 4-[4-(4-Fluorophenyl)-2-[4-(methylsulfinyl)phenyl]-1H-imidazol-5-yl] pyridine hydrochloride (SB203580), 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) and 2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one (LY 294002) were obtained from Ascent Scientific, Bristol, UK. Sodium fluoroacetate (FA) was obtained from Sigma-Aldrich, Poole, UK. Anti-phospho-p38 MAPK and anti-phospho-c-Jun N-terminal kinase (JNK) antibodies were obtained from Invitrogen, Paisley, UK. Anti-phospho-ERK, total anti-p38 MAPK, total anti-JNK and total anti-ERK antibodies were obtained from Santa Cruz Biotechnology, Heidelberg, Germany. All horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson Immuno-Research Laboratories, Newmarket, UK. Alexa Fluor anti-rabbit 488 and Alexa Fluor 555 anti-mouse, were obtained from Invitrogen (Paisley, UK). Anti-microtubule associated protein 2 (MAP2) was obtained from Sigma-Aldrich (Poole, UK) with anti-green fluorescent protein (GFP) obtained from Clontech (Mountain View, USA).

2.2 Preparation of organotypic hippocampal slice cultures.

Organotypic hippocampal slice cultures (OSCs) were prepared as described previously (Greenwood and Bushell, 2010). Briefly, C57BL/6 mice (P4-7) were killed by cervical dislocation, according to Home Office UK Schedule 1 guidelines, followed by decapitation into ice-cold 70% ethanol. The brain was removed, transferred to ice-cold preparation media (50% MEM, 25% Hank balanced salt solution, 25% horse serum, 25 mM D-glucose and 1
mM L-glutamine), bisected and glued to a sterile dissecting platform. Following submersion in ice-cold preparation media, parasagittal sections (300µm) were cut using a vibratome (Vibratome, Bannockburn, IL, USA) and the hippocampus removed. The slices were transferred to Millicel insert membranes (0.5 µm; Millipore Corporation, Bedford, MA, USA) and placed into 6-well culture plates containing pre-equilibrated preparation media (1 mL/well). These were incubated at 37°C / 5% CO₂ for 24 hours after which the preparation media was replaced with serum-free culture media (SF media; 98% Neurobasal-A; 2% B27 supplement and 2 mM L-glutamine) and maintained at 37°C / 5% CO₂. The culture media was then exchanged three times per week with all experiments performed on OSCs maintained for 13-15 days in vitro (DIV).

2.3 Lymphocyte preparation.

Peripheral lymph nodes were harvested from C57BL/6 mice (6-10 weeks old) and transferred to complete RPMI 1640 (cRPMI) media containing 10% foetal calf serum and 2mM L-Glutamine. The lymph nodes were homogenised and passed through a cell strainer (40 µm pore), transferred to a sterile tube and centrifuged at 400 x g for 5 minutes. The pellet was then washed at least 2 times in serum free (SF) media and the cells re-suspended in SF media at 1 X 10⁶ cells per ml, the final concentration used in all experiments. T cells were purified from mixed lymphocyte preparations using magnetic cell sorting (pan T cell isolation kit; Miltenyi Biotec, UK) following the manufacturer’s instructions. For lymphocyte preparations containing GFP-expressing T cells used to investigate cell-cell interactions, these were isolated from hCD2-GFP transgenic mice (Fumagalli et al., 2011). In experiments where T cell activation was required, non-purified lymphocyte preparations were stimulated with anti-mouse CD3e (1µg /ml) for 24 hours whereas purified T cell preparations were exposed to both anti-mouse CD3e (2µg /ml) and anti-mouse CD28 (3µg /ml) for 24 hours.
2.4 Assessment of neuronal death using propidium iodide staining.

OSCs (13-15 DIV) were checked under bright field microscopy for well-defined morphology prior to experimental use. All drugs and lymphocytes (1 X 10^6 cells mL^-1) were mixed in fresh culture media and equilibrated at 37°C / 5% CO₂. Conditioned media was prepared by centrifuging (2000 rpm, 10 minutes) media containing lymphocytes which were incubated for 18 hours at 37°C / 5% CO₂ and where appropriate, drugs were added directly to conditioned media. For assessment of neuronal death, OSCs were transferred to culture media containing propidium iodide (PI; 2 µM) following exposure to KA for 1 hour and incubated for 18 hours at 37°C / 5% CO₂. For oxygen glucose deprivation experiments, artificial cerebro-spinal fluid (aCSF) containing (in mM): 124 NaCl, 3 KCl, 26 NaHCO₃, 2.5 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂ and 10 sucrose was continuously bubbled with nitrogen for at least for 2 hours prior to OSCs being transferred to OGD-aCSF and incubated in a nitrogen chamber for 30 minutes with the OSCs then transferred into fresh PI containing media and incubated for 18 hours at 37°C / 5% CO₂. Where OSCs were incubated either with lymphocytes or conditioned media in the absence or presence of drugs, PI was added directly to the media and slices incubated for 18 hours at 37°C / 5% CO₂. Following treatment, OSCs were transferred to a microscope-mounted incubator chamber (37°C; Solent Scientific, Segensworth, UK) and PI fluorescent images were captured using a Nikon TE300 (Nikon, Kingston upon Thames, UK) inverted epi-fluorescent microscope (4X objective; Ex: 575/25 nm; Em: 626/28 nm) equipped with a Hamamatsu CCD camera (Hamamatsu Photonics, Welwyn Garden City, UK) controlled by Metamorph software (Molecular Devices Palo alto, CA, USA). The cell death was quantified by measuring the integrated density (mean fluorescence ÷ slice area) of PI fluorescent images using ImageJ software (NIH) as described previously (Greenwood and Bushell, 2010). A concentration-response curve was generated using KA (3 µM to 3 mM) to determine maximum neuronal death. As KA (3 mM, 1 hour) caused maximum neuronal death, all
neuronal death values were expressed throughout in terms of this maximum value. In the OGD-induced cell death, a single treatment protocol was used.

2.5 Cytokine array

Commercially available cytokine antibody array dot blots (Proteome Profiler™ Mouse Cytokine Array Panel A, R&D Systems Europe) were used to measure the relative amounts of cytokines in fresh media, media incubated for 18 h and lymphocyte-conditioned media. A detection antibody, each targeted to a specific cytokine, was added to the treated media samples. The cytokine antibody array dot blots were then incubated overnight at 4°C with the media. Following this, the media was washed from the blot and a Streptavidin-HRP solution was added to the membrane for 30 min at room temperature. The cytokine array dot blot was again washed and a chemiluminescent HRP substrate (Millipore) was added to the membrane. The dot blots were then imaged using the FujiFilm LAS-3000 imaging system. Images were captured as 8 bit grayscale TIFFs and the relative pixel density of each spot on the array was quantified using Image J software.

2.6 Western blotting

Western blotting was performed as previously described (Greenwood and Bushell, 2010). Briefly following treatment, OSCs were transferred to ice-cold HEPES-buffered saline and kept on ice. OSCs were washed three times with ice-cold phosphate buffer solution (PBS, 2ml) and then cut out from the insert membranes and transferred to solubilisation buffer and incubated on ice for at least 1 hour with occasional vortexing. Insert membranes were removed and OSCs were triturated with a syringe and needle. Total cellular protein concentration was estimated by Bradford reaction and samples were normalised to 0.444µg/µL by adding solubilisation buffer. Samples were further diluted by 25% by adding
sample buffer and boiled for 5 minutes to denature proteins. Samples were loaded in 10%
SDS-polyacrylamide gels and subjected to electrophoresis which was blotted onto a
nitrocellulose sheet. Non-specific binding was blocked by incubating blots with NaTT buffer
containing 3% bovine serum albumin (BSA) for 2 hours. Blots were then incubated overnight
with specific primary antibodies prepared in 0.2% BSA/NaTT then washed with NaTT buffer
for 2 hours before incubating with either horse-radish peroxidase-conjugated anti-mouse or
anti-rabbit IgG secondary antibody for 90 minutes. Blots were again washed for 2 hours and
developed using enhanced chemiluminescence (ECL) reagents with a X-OMAT developing
machine. After detection of phosphor-specific bands, blots were stripped using a stripping
solution for 1 hour on an orbital shaker. Blots were then washed for 30 minutes with NaTT
buffer. These blots were then re-blocked and re-probed with non-phospho-specific antibody
as described above.

2.6 Immunohistochemistry

OSCs were incubated with lymphocyte preparations containing GFP-expressing T cells for
18 hours after treatment with or without KA for 1 hour. To confirm detection of GFP-
expressing T cells, 1 X 10^6 lymphocytes were added on the top of slices as opposed to the
usual arrangement of T cells underneath the transwell membrane. After 18 hours, the media
was removed by suction and 1 ml cold 4% paraformaldehyde (PFA) was added above and
below the insert membrane for 5 minutes. OSCs were washed twice with cold PBS and 100%
methanol was then added above and below the membrane for 10 minutes. Following PBS
washes, OSCs were incubated overnight with permeabilization solution (0.5% Triton X-100
in PBS) before blocking with 20% Bovine serum albumin in PBS for 4 hours at room
temperature. Anti-MAP2 (1:5000) and anti-GFP (1:400) antibodies were then applied in 5%
BSA blocking solution to the OSCs overnight at 4^0 C. OSCs were then washed with blocking
solution and appropriate fluorescent secondary antibodies (Alexa488 and Alexa555) applied at room temperature (3 hours). OSCs were then washed with 5% blocking solution and mounted, using Vectashield, on a glass microscope slide. Z stack images were captured using a Leica TCS SP-5 laser scanning confocal system with a 40x oil immersion lens and optimised using Adobe Photoshop (version 7.0).

2.7 Data analysis

All data are presented as mean ± S.E.M. with the number of slices indicated for all experiments performed on at least four different culture preparations. The differences between groups were analysed using a Student’s unpaired t-test or one-way ANOVA with Tukey’s post-hoc analysis as appropriate. In all analyses, p< 0.05 were considered as significant.
3. Results

3.1 Lymphocytes are not toxic to OSCs.

As it is unclear whether lymphocytes play either a protective or pathogenic role in the CNS, we first sought to determine whether lymphocytes were neurotoxic to OSCs under our experimental conditions. Initially, OSCs were treated with a range of concentrations of KA (3 µM – 3mM) for 1 hour before staining with propidium iodide (PI) to determine the maximum cell death. As per previous studies in our laboratory (Greenwood and Bushell, 2010), KA induces neurotoxicity in a concentration-dependent manner with maximum cell death observed at 3mM (Figure 1A & B). Hence in all further experiments, OSCs were treated with KA (300 µM, 60 min) as a positive control of neurotoxicity with all levels of cell death being expressed as a percentage of this 3mM KA maximum. This toxicity was completely abolished in the presence of both the AMPA/KA antagonist, NBQX (20 µM), and the NMDA antagonist, DL-AP5 (100 µM; n ≥ 4; p < 0.001 compared to KA (300 µM) alone; Figure 1C). In contrast to the effects of KA, mixed lymphocyte preparations did not induce neurotoxicity in OSCs when exposed to lymphocytes for up to 18hours (P>0.05 compared to control, Figure 1A & C). This lack of neurotoxicity was evident irrespective of T cell activation, since OSCs exposed to T cells in the presence (18h, n=17) or absence (18h, n=9) of anti-CD3e stimulation displayed no evidence of cell death (Figure 1C). Together, these data demonstrate that lymphocytes are not directly neurotoxic to OSC in our mixed cell culture system.

3.2 Lymphocyte preparations exhibit neuroprotection against KA-induced cell death.

Having established that lymphocyte preparations are not neurotoxic to OSCs, we investigated whether OSC exposure to lymphocytes modulates KA-induced excitotoxicity. The presence
of lymphocytes for only 1 hour during initial treatment with KA (300 μM, 1h) resulted in similar levels of OSC toxicity as KA alone and this was independent of T cell activation state (non-activated T cells, n=16, P>0.05 compare to KA alone; activated T cells, n=14, P>0.05 compare to KA alone, Figure 2B). In contrast, significant neuroprotection was observed when OSCs were co-cultured with lymphocytes for 18 hours following an initial exposure to KA (300 μM, 1h). This lymphocyte-mediated neuroprotection was independent of T cell activation as KA-induced toxicity was reduced from 67.5 ± 5.6% (n=30) to 50.8 ± 3.8% (n=48, P<0.05 compared to 300 μM KA alone, Figure 2A & B) and 45.4 ± 3.4% (n=50, P<0.01 compared to 300 μM KA alone, Figure 2A & B) for preparations containing non-activated and activated T cells respectively. Thus, in our model of KA-induced excitotoxicity, lymphocytes are able to exhibit neuroprotection and this is independent of T cell activation.

3.3 Purified T cells exhibit neuroprotection against KA-induced cell death.

Having determined that a mixed lymphocyte population is neuroprotective in our experimental system, we next sought to determine the cell type responsible for mediating protection. Using flow cytometry, we confirmed that approximately 70% of the cells in our lymphocyte preparations were T cells (68.5 ± 1.8 %, n = 16). Therefore, to investigate the role of T cells in the observed protection against KA-induced neuronal death, T cells were purified from mixed lymphocyte preparations. In agreement with our initial findings, mixed lymphocyte preparations significantly reduced cell death in OSCs from 66.8 ± 2.7% (n=10) to 31.6 ± 3.1% (n=8, P<0.001 compared to 300 μM KA alone, Figure 2C). Similarly, T cells purified from the same lymphocyte preparation significantly reduced KA-induced neuronal death, an effect that was independent of T cell activation state with cell death being reduced to 42.7 ± 2.9% (n=9, P<0.001 compared to 300 μM KA alone, Figure 2C) and 51.7 ± 5.8%
(n=6, P<0.05 compared to 300 μM KA alone, Figure 2C) for non-activated and activated purified T cells respectively.

3.4 Soluble mediators underlie lymphocyte-induced neuroprotection.

Having established that lymphocyte preparations and purified T cells are neuroprotective under our experimental conditions, we investigated the mechanisms underlying the observed neuroprotection. To determine whether soluble mediators underlie lymphocyte-mediated neuroprotection, lymphocyte conditioned media (LCM) was assessed for protection against KA-induced neurotoxicity. Lymphocytes were cultured for 18 hours alone or in the presence of OSCs and the purified conditioned media transferred to KA-treated OSCs. Cell death in OSCs maintained in LCM for 18 hours after initial exposure to KA was significantly reduced irrespective of whether LCM was purified from mixed cultures of lymphocytes and OSC or from lymphocytes alone, suggesting that soluble mediators are responsible for the neuroprotective effects and that these are solely produced by lymphocytes (Figure 3A & B). In contrast, the neuroprotective effect of LCM was abolished when the media was boiled (63.4 ± 4.8%, n=7, P>0.05 compared to 300 μM KA alone, Figure 3A & B), suggesting the neuroprotective factor in LCM is protein in nature. Furthermore, to confirm that lymphocytes were essential for the LCM-mediated neuroprotection, conditioned media (CM) taken from KA treated slices (61.8 ± 3.8%, n=9, P>0.05 compared to 300 μM KA alone) and CM from primary hippocampal cultures (61.1 ± 3.6%, n=10, P>0.05 compared to 300 μM KA alone) were ineffective against KA-induced neurotoxicity. Further confirmation that direct cell-cell interaction is not required for the observed neuroprotection was achieved by utilising lymphocyte preparations containing GFP-expressing T cells isolated from hCD2-GFP transgenic mice in order that any T cell infiltration of the OSCs could be easily visualised. Examination of slices, either with or without the exposure of KA, maintained for
18 hours in the presence of lymphocyte preparations containing GFP-expressing T cells under conditions identical to those described above, revealed no infiltration of T cells into the OSCs although GFP-expressing T cells were observed when applied directly to the slice (data not shown). These data strongly suggest that soluble mediators released from lymphocytes are responsible for the observed neuroprotection, with no cell-cell interaction required. In order to try and identify the soluble mediator(s) involved, arrays were performed to analyse the levels of 40 cytokines found in fresh media, media incubated for 18 hours and LCM that was prepared as per the neuroprotective experiments described earlier. These cytokine arrays revealed that a robust increase in IL-16 levels were evident in LCM compared to fresh and incubated media (Figure 3C & D) with the level of all other cytokines investigated being similar in all three media preparations indicating that IL-16 may be the soluble mediator underlying the neuroprotective effects observed in the current experiments.

3.5 Lymphocyte mediated neuroprotection involves astrocytic activation and inhibition of ERK and p38 MAP kinase signalling.

Despite having identified IL-16 as the potential cytokine underlying the lymphocyte-mediated neuroprotection, the cell type and signalling pathways within the OSCs on which it acts to produce this effect is unknown. Hence we next utilised the glial metabolic inhibitor, fluoroacetate (FAc), which we have successfully used to investigate astrocyte function in previous studies (Greenwood and Bushell, 2010; Gan et al., 2011). Exposure of OSCs to FAc (10 μM, 3h) did not affect KA-induced cell death (n=12, P>0.05 compared to 300 μM KA alone, Figure 4A). However, neuroprotection elicited by LCM (50.7 ± 5.2%, n=15, P<0.01 compared to 300 μM KA alone) was significantly reduced in FAc-treated OSCs (66.2 ± 2.8%, n=10, P<0.001 compared to 300 μM KA + LCM, Figure 4A), suggesting that astrocytes are important in the neuroprotection induced by lymphocytes. We then examined
whether common signalling pathways that are known to be modulated by numerous immunological mediators, namely MAPKs, are involved in the lymphocyte-mediated neuroprotection. Initial experiments were performed to determine if and how MAPK activity was modulated following KA-induced cell death in the absence and presence of lymphocytes. However, exposure of OSCs to KA led to considerable variability in the MAPK activity between slices and experiments and thus we decided to determine the effect on MAPK activity of subjecting untreated OSCs to lymphocytes. Exposure of OSCs to lymphocytes (18h) resulted in a significant reduction in ERK and p38 MAPK activity to 66.2 ± 6.1 % of control (n=10, P<0.05 compared to control, Figure 4B & C) and 64.1 ± 4.5% of control (n=6, P<0.05 compared to control, Figure 4B & C). In contrast, JNK 1/2 activity was unaffected by exposure to lymphocytes (108.6 ± 12.7% of control, n=10, P>0.05 compared to control, Figure 4B & C). If this lymphocyte-mediated reduction in ERK and p38 MAPK activity in untreated slices contributed to the observed lymphocyte-mediated neuroprotection against KA-induced cell death, the pharmacological inhibition of both kinases should also be protective against KA-induced neurotoxicity. Indeed, co-incubation with the p38 MAPK inhibitor, SB203580 (100μM), and the upstream ERK inhibitor, U0126 (20μM) significantly reduced KA-induced cell death from 71.8 ± 2.8 (n=15) to 53.1 ± 1.1 (n=11, P<0.001 compared to 300 μM KA, Figure 4D). Having shown a role for MAPK inhibition, we also investigated whether the PI3K pathway is involved in lymphocyte-mediated neuroprotection. To negate any issues regarding PI3K inhibition on lymphocyte function, pharmacological inhibition of PI3K using LY294002 (10μM) was performed in the presence of lymphocyte conditioned media (LCM). LCM significantly reduced KA-induced cell death from 71.8 ± 2.8 (n=15) to 53.3 ± 2.0 (n=11, P<0.01 compared to 300 μM KA, Figure 4D) and this was unaffected by treatment with LY294002 (56.6 ± 2.2%, n=13, P>0.05 compared to 300 μM KA + LCM, Figure 4D). In the absence of LCM, no change in KA-induced toxicity was
observed in the presence of LY294002 (68.7 ± 1.6%, n=6, P>0.05 compared to KA alone, Figure 4D).

3.6 Lymphocytes are protective against OGD-induced toxicity.

Having established the neuroprotective effect of lymphocytes against KA-induced toxicity, we further investigated whether lymphocytes protect OSCs against another in vitro model of neurotoxicity, OGD. OGD (30 mins) induced significant cell death in OSCs and exposure to lymphocytes reduced the neurotoxicity to 61.6 ± 3.0 % of OGD control (n=19, P<0.001 compared to control, Figure 5A & B). LCM also significantly reduced OGD-induced cell death to 65.9 ± 7.4 % of OGD control (n=5, P<0.05 compared to control, Figure 5B) indicating that in agreement with the KA-induced toxicity experiments, soluble mediators released by lymphocytes are responsible for the observed neuroprotection against OGD. However, in contrast to the KA experiments, pre-treatment of OSCs with FAc (10μM, 3hr) had no significant effect LCM-mediated neuroprotection (56.8 ± 6.0 % of OGD control, n=6, P>0.05 compared to LCM alone, Figure 5B), a finding which suggests that astrocytes do not play a significant role in the protection against OGD. However, similar signalling mechanisms appear to underlie the observed neuroprotection as co-incubation with SB203580 (100μM), and U0126 (20μM) significantly reduced OGD-induced toxicity to 66.3 ± 5.9 % of control (n=9, P<0.01 compared to OGD control, Figure 5B) whereas LY294002 (10μM) was without effect (72.5 ± 1.6% of OGD control, n=6, P>0.05 compared to LCM alone, Figure 5B).
4. Discussion

In the present study, we show that lymphocytes are protective in two *in vitro* models of neurotoxicity and that protection is independent of T cell activation. We suggest that the lymphocyte-mediated protection involves soluble mediators, presumably IL-16 and, at least in the case of KA-induced toxicity, is mediated indirectly via astrocytes. In addition, we propose that an inhibition of both p38 MAPK and ERK activity contributes to the observed neuroprotection.

4.1 Lymphocytes are neuroprotective independent of T cell activation state.

The role of the immune system in modulating the CNS under physiological and pathophysiological conditions is an area of intense interest. Classically, activation of the immune system and recruitment of immune cells was thought to contribute to and exacerbate pathophysiological conditions within the CNS. Indeed, in the archetypal autoimmune CNS disease, multiple sclerosis, it is established that lymphocytes invade the CNS and contribute to the disease neuropathology (Larochelle et al., 2011; Stadelmann, 2011) and a role for neuroinflammation has been implicated in numerous CNS diseases including Alzheimer’s disease (Grammas, 2011) and Parkinson’s disease (Barnum and Tansey, 2010; Qian et al., 2010). However, studies also suggest that lymphocytes may have a neuroprotective role in the CNS when investigated using models of spinal cord injury (Yoles et al., 2001; Sroga et al., 2003; Beck et al., 2010), facial nerve axotomy (Serpe et al., 1999; Sanders and Jones, 2006; Xin et al., 2011) and ischaemia (Frenkel et al., 2005; Liesz et al., 2009). This latter role for lymphocytes reflects our finding in the present study that lymphocytes are not neurotoxic to OSCs under our experimental conditions but are protective against KA-induced and OGD-induced toxicity. Our findings are in agreement with a previous study which focused specifically on the consequence of exposing organotypic cultures to T cell subtypes.
(Wolf et al., 2002). It revealed that both Th1 and Th2 cells were protective in a contact independent manner. However in contrast to our own study, increased neuroprotection was observed following T cell activation and protection was against secondary damage initiated by explantation of tissue from living animal to the culture dish. Conversely, our study utilised established models of neurodegeneration and experimental ischaemia to assess lymphocyte-mediated neuroprotection (Noraberg et al., 2005; Wang et al., 2005; Zheng et al., 2011). Hence we have shown for the first time that lymphocytes are protective in established in vitro models of neurodegeneration and ischaemia. Furthermore, in our hands, this neuroprotection is independent of the T cell activation state with lymphocyte-mediated neuroprotection being similar whether or not they contained activated T cells. This was also true when purified T cells were utilised, with both activated and non-activated purified T cells being protective against KA-induced neurotoxicity. The discrepancy in the role of T cell activation state between our studies could simply be due to the experimental parameters used to investigate T cell function, but further investigation into this is beyond the scope of this study.

As stated previously, the finding that T cells were neuroprotective is in agreement with previous studies where T cell function has been investigated in in vivo models of neurodegeneration (Serpe et al., 1999; Yoles et al., 2001; Sroga et al., 2003; Frenkel et al., 2005; Sanders and Jones, 2006; Liesz et al., 2009; Beck et al., 2010; Xin et al., 2011). However, it should be noted that many of these studies were performed in immune compromised animals and as such, it could be argued that the observed effects may be due to T cell independent broader effects of development in the absence of a functioning adaptive immune system (Rook et al., 2011). With regard to T cell subtypes and neuroprotection, recent studies have implicated Treg cells as being the key lymphocyte population involved in neuroprotection in experimental stoke (Liesz et al., 2009 but also see Ren et al., 2011).
Similarly, in experimental autoimmune encephalitis (EAE) models, neurons have been proposed to induce Treg cells from encephalitogenic T cells and these Treg cells function to suppress EAE (Liu et al., 2006). In addition to Treg, Th2 cells have been suggested to play a role in neuroprotection (Hendrix and Nitsch, 2007; Yong and Marks, 2010) but there are discrepancies as to whether this occurs in a contact-dependent (Roy et al., 2007) or contact-independent manner (Wolf et al., 2002). Our findings support the suggestion that T cells can be neuroprotective and that protection is via soluble factors; however which T cell subtype is responsible for these effects under our experimental conditions requires further investigation.

4.2 Lymphocyte-mediated neuroprotection is contact-independent.

Having established that lymphocyte preparations are neuroprotective in a contact-independent manner, we also sought to identify the underlying mechanisms. We confirmed that soluble mediators were responsible for the observed neuroprotection as lymphocyte-conditioned media was protective against KA-induced and OGD-induced toxicity. Furthermore, as boiling the lymphocyte-conditioned media abolished this neuroprotection, we conclude that protein(s) within the media underlie this neuroprotection. We performed an array of 40 cytokines in order to try and identify the cytokine(s) potentially underlying this neuroprotection with our results revealing a robust increase in IL-16 levels only in media conditioned in the presence of lymphocytes with all other cytokines investigated being at similar levels in all three conditions examined. IL-16 was originally described as a T cell chemoattractant factor but it is now accepted as a cytokine as it lacks the classic chemokine motifs (Center and Cruickshank, 1982; Cruickshank and Center, 1982; Bacon et al., 2002). In T cells, IL-16 has been shown increase intracellular Ca\(^{2+}\), inositol 1,4,5-triphosphate levels and stress activated protein kinase activity as well as stimulate the release of proinflammatory cytokines (Cruickshank et al., 1991; Krautwald, 1998; Zhang et al., 2008); whether IL-16
elicits similar changes in CNS cell types remains unknown. However, a neuronal IL-16 has also been described and it is proposed that this neuronal form may play some autocrine function within the CNS (Kurschner and Yuzaki, 1999) by interacting with CD4 which has been shown to be expressed in neurons (Omri et al., 1994). Regarding the role of IL-16 in CNS diseases, evidence exists relating increased levels of IL-16 to disease severity in the EAE mouse model (Skundric et al., 2005) and increased microglial expression following trimethyltin neurotoxicity (Guo et al., 2004) indicating that elevated IL-16 levels are linked to detrimental events. In contrast to this, recent evidence suggests that IL-16 may play a neuroprotective role by interacting with its binding partner, CD4 and its co-receptor CCR5 as these have been shown to be expressed in several cell types within the CNS (Omri et al., 1994, Sorce et al., 2011). Indeed, CCR5 has been shown to be upregulated by a number of neurotoxic insults including kainic acid (Chen et al., 2003). Furthermore, motor neuron death is accelerated in the absence of CCR5 (Gamo et al., 2008) and CCR5 deficient mice have reduced infarct size indicating a protective role of this receptor (Sorce et al., 2010) but whether this is mediated via glia cells or a direct action on neurons is unknown. Therefore it remains speculation as to whether the neuroprotection observed in the present study is mediated via IL-16 acting directly on cells within the OSC or via stimulating the release of other factors from lymphocytes not detected in our array but our data indicates that the neuroprotective effects revealed in this study involves IL-16 but the exact mechanisms require further investigation.

4.3 Neuroprotection requires astrocytic activation and MAPK inhibition.

Having identified a potential specific soluble mediator that underlies the lymphocyte-mediated neuroprotection under our experimental conditions, we then chose to determine whether they interacted with a specific cell type within OSCs to induce the neuroprotection.
OSC are made up of a heterologous population of cell types including astrocytes, microglia and neurons. Hence, due to the recent increase in our understanding of astrocytes function within the CNS under both physiological and pathophysiological conditions (Bélanger and Magistretti, 2009; Rodriguez and Verkhratsky, 2011) and having established that astrocytes were involved in the neuroprotection observed against KA-induced neurotoxicity in OSCs in a previous study (Greenwood and Bushell, 2010), we investigated whether inhibition of astrocytic activity would alter the lymphocyte-mediated neuroprotection using techniques established in our laboratory (Greenwood and Bushell, 2010; Gan et al., 2011). Inhibition of astrocytic activity using the glial toxin, FAc, abolishes lymphocyte-mediated neuroprotection against KA-induced neurotoxicity but not in the OGD model. Until recently, the primary role of astrocytes in the normal brain was thought to be maintaining the microenvironment of neurons by performing roles including glutamate uptake and recycling, pH and K+ buffering as well as supply of energy substrates (Bélanger and Magistretti, 2009; Rodriguez and Verkhratsky, 2011) whereas it is now proposed that astrocytes can play a key role in modulating synaptic transmission and plasticity (Bélanger and Magistretti, 2009; Perea et al., 2009; Barker and Ullian, 2010; Rodriguez and Verkhratsky, 2011). Furthermore, astrocytes have recently come under scrutiny regarding their role in neurodegeneration associated with certain CNS disorders including Alzheimer’s disease and Parkinson’s disease (Bélanger and Magistretti, 2009; Halliday and Stevens, 2011; Rodriguez and Verkhratsky, 2011). However, there is debate as to whether astrocytes are either beneficial or detrimental in CNS diseases and this may depend on how far the disease has progressed and / or on which mediators are released at that stage of the disease. Astrocytes are capable of releasing anti- and / or pro-inflammatory mediators and it may depend on the relative relationship of these which determines the eventual contribution of astrocytes to the disease. Under our experimental conditions, astrocytes clearly play a role in the observed neuroprotection against KA-induced
toxicity as shown by the loss of neuroprotection when astrocytic function is inhibited using FAc. The ability of astrocytes to release neurotrophins is thought to contribute to their role in neuroprotection with for example, BDNF and glial derived neurotrophic factor (GDNF) known to be neuroprotective in models of neurodegenerative disorders for many years (Spina et al., 1992; Tomac et al., 1995). Indeed, GDNF has been the subject of intense investigation in recent times regarding its potential therapeutic use in Parkinson’s disease (Mickiewicz et al., 2011; Safi et al., 2011). However, whether astrocytically released neurotrophins do indeed underlie the lymphocyte-mediated neuroprotection in the present study requires further investigation especially as it has been suggested that T cell induced release of neurotrophins from astrocytes is contact-dependent (Roy et al., 2007). In contrast to the role astrocytes play in lymphocyte-mediated neuroprotection against KA-induced toxicity, our data indicates that astrocytes do not contribute to lymphocyte-mediated protection against OGD-induced toxicity. The sensitivity of astrocytes to ischaemic insults has long been an area of debate with astrocytes from different brain regions showing differential sensitivity to ischaemia and in vitro models of ischaemia revealing that despite both astrocytes and neurons being sensitive to OGD, astrocytic death is delayed in comparison to neuronal death (Zhao and Flavin, 2000; Xu et al., 2001; Giffard and Swanson, 2005; Ouyang et al., 2007). In contrast, astrocytic dysfunction is proposed to contribute to ischaemia-induced loss of neurons in in vivo models of forebrain ischaemia (Chen et al., 2005; Giffard and Swanson, 2005). This divergence in the role of astrocytes may indicate that OGD toxicity is not cell type selective whereas KA induces toxicity primarily in neurons and therefore astrocytes do not contribute to the neuroprotection against OGD because they themselves are dysfunctional or dead. Despite this, it is clear that lymphocytes are protective in two different in vitro neurodegenerative models and the soluble mediators involved may have direct actions resulting in neuroprotection either directly or indirectly via astrocytes.
Having shown that lymphocytes were neuroprotective in two different *in vitro* neurodegenerative models, we sought to investigate the signalling pathways that may underlie this. We focussed our efforts on the MAPK and PI3K pathways as it is well established that cytokines can modulate both these pathways (Schieven, 2009; Kim and Choi, 2010; Kim and Suresh, 2013) and that modulators of the MAPK pathway in particular are therapeutic targets for the CNS neurodegenerative disorders (Cuny, 2009; Yasuda et al., 2011). Exposure of OSCs to lymphocytes reduced p38 MAPK and ERK activity whereas no effect on JNK 1/2 activity was observed. Significantly, when the reduction in MAPK activity was mimicked using pharmacological inhibitors, a similar level of neuroprotection was observed. As the lymphocyte-mediated neuroprotection is contact-independent, these data indicate that the soluble mediators released by lymphocytes results in this reduction in MAPK activity. Of the potential candidates, this reduction in activity would suggest that neurotrophins do not underlie the protection as studies have shown they generally lead to the activation of MAPK pathways via Trk receptors (Ouwens et al., 2002; Shi et al., 2005; Reichardt, 2006). Despite this, it is interesting to note that the neuroprotection mediated by lymphocytes involves an inhibition of p38 MAPK as increased p38 MAPK activity has been associated with several CNS neurodegenerative disorders and inhibitors of its activity are proposed as therapeutic drugs for CNS disorders (Cuny, 2009; Yasuda et al., 2011). In contrast to the role of p38 MAPK in neurodegeneration, it is generally thought that ERK activation is neuroprotective (Mehta et al., 2007) however there is a growing body of evidence which suggests that increased and/or prolonged ERK activation may indeed contribute to cell death and CNS diseases (Zhuang and Schnellmann, 2006). Hence it is feasible that a reduction in ERK activity as observed in the present study would be advantageous in terms of cell survival. Finally, our finding that JNK 1/2 activity is
unaffected by exposure to lymphocytes suggests that modulation of this pathway does not appear to contribute to the observed lymphocyte mediated neuroprotection.

5. Conclusions

In conclusion, our data demonstrates that lymphocytes are neuroprotective against KA-induced and OGD-induced toxicity under our experimental conditions and that this is independent of T cell activation. We propose that this neuroprotection is mediated indirectly by the action of a soluble mediator, likely to be IL-16, on astrocytes and the inhibition of ERK and p38 MAPK activities. These data, along with previous studies, suggest a neuroprotective role for lymphocytes in models of neuronal death or injury, and that lymphocytes may play multiple roles in CNS disorders. However, further investigation is required to decipher when and why the divergence between their proposed protective or detrimental roles occur in vivo.
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References
Bacon, K., Baggioni, M., Broxmeyer, H., Horuk, R., Lindley, I., Mantovani, A.,
Maysushima, K., Murphy, P., Nomiyama, H., Oppenheim, J., Rot, A., Schall, T., Tsang, M.,
Batoulis, H., Recks, M.S., Addicks, K., Kuerten, S., 2011. Experimental autoimmune
tencephalomyelitis--achievements and prospective advances. APMIS 119, 819-30.
Becher, B., Bechmann, I., Greter, M., 2006. Antigen presentation in autoimmunity and CNS
2010. Quantitative analysis of cellular inflammation after traumatic spinal cord injury:
evidence for a multiphasic inflammatory response in the acute to chronic environment. Brain
133, 433-47.
Neurosci. 11, 281-95.
Center, D.M., Cruickshank, W., 1982. Modulation of lymphocyte migration by human
lymphokines. I. Identification and characterization of chemoattractant activity for
not a necessary inflammatory mediator in kainic acid-induced hippocampal injury: evidence


Sorce, S., Bonnefont, J., Julien, S., Marq-Lin, N., Rodriguez, I., Dubois-Dauphin, M.


Yong, V.W., Marks, S., 2010. The interplay between the immune and central nervous systems in neuronal injury. Neurology 74 Suppl 1,S9-S16.


Figure Legends

Fig 1. Lymphocytes do not induce neurotoxicity in organotypic slice cultures. A. Representative images showing propidium iodide fluorescence 18h after exposure to KA (30 µM and 300 µM, 1 h) or mixed lymphocytes (1h). B. KA induces neuronal cell death in OSCs in a concentration-dependent manner. C. Summary illustrating the effects of lymphocyte preparations containing activated and non-activated T cells on neuronal survival in OSCs. Data are expressed as mean ± S.E.M. and are normalised with respect to 3mM KA. ***P<0.001 versus control. Scale bars: 150 µm.

Fig 2. Lymphocyte-mediated neuroprotection against KA-induced neurotoxicity is independent of T cell activation state. A. Representative images illustrating cell death 18h after exposure to KA alone (300 µM, 1h) or KA followed by exposure to lymphocytes containing activated or non-activated T cells (18h). B. Bar chart revealing that lymphocyte preparations were ineffective against KA-induced neurotoxicity when OSCs were exposed to lymphocytes for 1h post insult whereas neurotoxicity was significantly reduced in OSCs exposed to lymphocytes for 18 h post insult with this protection being independent of T cell activation state. C. Summary showing that protection against KA-induced neurotoxicity is observed with purified T cells, independent of activation state and T cell depleted lymphocyte preparations. Data are expressed as mean ± S.E.M. and are normalised with respect to 3mM KA. *P<0.05, **P<0.01, ***P<0.001 versus control. Scale bars: 150 µm.
Fig 3. Soluble mediators underlie lymphocyte-mediated neuroprotection. A. Representative images illustrating cell death 18 h after exposure to KA alone (300 µM, 1h) and in the presence of lymphocyte conditioned media (LCM) or boiled LCM for 18h post insult. B. Summary illustrating that LCM is neuroprotective against KA-induced toxicity and the conditioning is not dependent on the presence of OSCs. In contrast, boiled LCM or conditioned media (CM) from KA treated OSCS or primary hippocampal cultures are without effect. C. Representative array blot for IL-16 revealing a robust increase in IL-16 in LCM compared to media controls. D. Scatter plot showing IL-16 values from the primary screening of 40 cytokines. No difference was seen in the other 39 cytokines assayed. Data are expressed as mean ± S.E.M. and are normalised with respect to 3mM KA. **P<0.01 versus control. ###P<0.001 versus KA + LCM. Scale bars: 150 µm.

Fig 4. Astrocytic activation and inhibition of MAPK activity underlies lymphocyte-mediated neuroprotection. A. The glial toxin, FAc (10 µM) does not reduce KA-induced toxicity but abolishes LCM-mediated neuroprotection. B. Representative blots illustrating that exposure to lymphocytes reduces p-p38 MAPK and p-ERK but not total protein in OSCs. No effect on p-JNK 1/2 or total JNK was observed. C. Bar chart revealing the effect of lymphocytes on p38 MAPK, ERK and JNK 1/2 activity in OSCs. D. Summary illustrating that the pharmacological inhibition of p38 MAPK and ERK with SB203580 and U0126 is neuroprotective against KA-induced neurotoxicity whereas inhibition of PI3K was without effect on LCM-mediated neuroprotection. Data are expressed as mean ± S.E.M. and are normalised with respect to 3mM KA. *P<0.05, **P<0.01, ***P<0.001 versus control, ####P<0.001 versus LCM alone. Scale bars: 150 µm.
Fig 5. Lymphocytes are neuroprotective against OGD-induced cell death.

A. Representative images illustrating cell death in OSCs 18 h after exposure to OGD (30 mins) either alone or maintained in the presence of lymphocytes. B. Summary showing that lymphocytes and LCM are protective against OGD-induced cell death, an effect which is mimicked by p38 MAPK and ERK inhibition but not by PI3K inhibition. Data are expressed as mean ± S.E.M. and are normalised with respect to 3mM KA. *P<0.05, **P<0.01, ***P<0.001 versus control. Scale bars: 150 µm.
Figure 1

(A) KA concentration (M)

(KA (30 μM)    KA (300 μM)    Mixed lymphocytes)

(B) Cell death (%)

(C) Cell death (%)

Control    Mixed lymphocytes    Anti-CD3 activated mixed lymphocytes    KA    KA + AP5 + NBQX
Figure 2

A

KA

Alone + lymphocytes + lymphocytes inc.
(18 hr) (18 hr) activated T cells

B

Cell death (%)

KA alone KA + lymphocytes KA + CD3 activated
lymphocytes

Alone 1hr 18hr 1hr 18hr

KA (300 μM)

C

Cell death (%)

Alone lymphocytes T cells Activated T cells

KA (300 μM)
Figure 3

A

KA

Alone + LCM + boiled LCM

B

Cell death (%)

0 10 20 30 40 50 60 70 80 90

Alone LCM (+ OSC) LCM (- OSC) LCM (boiled) CM (KA) CM (PHC)

KA (300 μM)

C

D

IL-16 Array Set A (arbitrary units)

Control LCM Media (18h)

IL-16 Array Set B (arbitrary units)
Figure 4

A

- lymph + lymph - lymph + lymph - lymph + lymph

Phospho

Total

p38 MAPK ERK JNK

B

MAPK phosphorylation (% of control)

C

Cell death (%)

vehicle SB203580 + U0126 LY294002 alone LCM LCM + LY294002

KA (300 μM)

D

Cell death (%)

Alone FAc LCM LCM + FAc

KA (300 μM)
Figure 5

A

OGD

**Alone**

+ lymphocytes (18 hr)

B

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cell death (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>60 ± 4</td>
</tr>
<tr>
<td>SB203580 + U0126</td>
<td>66 ± 3</td>
</tr>
<tr>
<td>LCM</td>
<td>70 ± 6</td>
</tr>
<tr>
<td>LCM + LY294002</td>
<td>75 ± 4</td>
</tr>
<tr>
<td>LCM + FAC</td>
<td>65 ± 3</td>
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* * * * *

OGD (30 mins)