Lymphocytes Protect Cortical Neurons Against Excitotoxicity Mediated by Kainic Acid, an *in vitro* Model for Neurodegeneration

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ABSTRACT

Background

Neurodegenerative disease is a progressive loss of neurons from the central nervous system (CNS). Various conditions have been implicated for such conditions including ageing, inflammation, stress and genetic predisposition. Recently, studies have linked neurodegeneration with inflammation. Some studies have suggested the harmful effect of immune response while others have argued its neuroprotective role in neurodegeneration of the CNS. However, the precise role of inflammation and immune cells in such condition is still not clear.

Objective

To investigate the role of lymphocytes in neurodegeneration of the CNS and determine the underlying mechanism.

Method

We have used 4-7 days old mouse pups (C57Bl6) to prepare organotypic slice cultures which were cultured for 13-15 days prior to experiment. To induced cell death kainic acid was used and considered as an in vitro model for neurodegeneration. Lymphocytes were obtained from peripheral lymph nodes of 5-10 weeks old adult mouse which were used in the current study. Propidium iodide was used as a fluorescent dye to determine cell death in brain slice cultures.

Result

Lymphocytes do not induce cell death in slice cultures in the absence of any toxic insult whereas, after applying toxic insult to the slice cultures using kainic acid, lymphocytes show neuroprotection against such insult. Similarly, purified non-activated and purified activated T cells along with T cells depleted lymphocyte preparation also exhibit neuroprotection against kainic acid-induced cell death. We further, have demonstrated that the observed neuroprotection is contact-independent and soluble mediators released from lymphocytes are responsible for the observed neuroprotection. Moreover, our study has revealed that soluble mediators exhibiting neuroprotection act via astrocytes.

Conclusion

Lymphocyte preparations are neuroprotective and the observed neuroprotection is contact-independent. Soluble mediators released from lymphocytes are responsible for the observed neuroprotection.

KEY WORDS

Astrocytes, lymphocytes, neurodegeneration, neuroprotection, t cells

INTRODUCTION

Neurodegenerative disease is a progressive loss of neurons from the central nervous system (CNS) associated with a deficit in the function of the affected region. Various conditions have been suggested in neurodegeneration of the CNS including ageing, inflammation, stress and trauma and genetic predisposition.[1-4] Recent researches have shown a strong link between inflammation and neurodegeneration.[5,6] However, the role of inflammation and immune cells in such condition is still elusive.

In this study, we have investigated the role of immune cells in neurodegenerative condition of the CNS using organotypic brain slice for the possible therapeutic intervention. The kainic acid-induced cell death was taken as a model for neurodegeneration.[7]

METHODS

(2S,3S,4S)-3-(carboxymethyl)-4-(prop-1-en-2-yl) pyrrolidine-2-carboxylic acid (Kainic acid, Kainate), 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) and 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) were obtained from Ascent Scientific, Bristol, UK. Sodium fluoroacetate (FA) was obtained from Sigma-Aldrich, Poole, UK.

Preparation of organotypic brain slice cultures

Organotypic brain slice cultures (OSCs) were prepared as described previously.[8] C57Bl6 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, 25mM D-glucose and 1mM L-glutamine), bisected and glued to a sterile dissecting platform. Following submersion in ice-cold preparation media, parasaggital sections (300µm) were cut using a vibratome (Vibratome, Bannockburn, IL, USA) and the cortico-hippocampal part was dissected out. The slices were transferred to Millicel insert membranes (0.5µm; Millipore Corporation, Bedford, MA, USA) and placed into 6-well culture plates containing pre-equilibrated preparation media (1ml/well). These were incubated at 37^0C/5% CO2 for 24hours after which the preparation media was replaced with serum free media (culture media; 98% Neurobasal-A; 2% B27 supplement and 2mM L-glutamine) and maintained at 37^0C/5% CO_2. The culture media was then exchanged three times per week with all experiments performed on OSCs maintained for 13-15 days in vitro (DIV).

Lymphocyte preparation

Peripheral lymph nodes were harvested from C57Bl6 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 triturated and passed through a cell strainer (40µM pore), transferred to a sterile tube and centrifuged at 1400 rpm for five minutes. The pellet was then washed at least two times in culture media and the cells were re-suspended in culture media, counted in the presence of trypan blue to identify viable cells and diluted to $1X10^6$ cells per ml, the final concentration used in all experiments. T cells were purified from lymphocyte preparations using magnetic-assorted cell sorting (MACS) and depleting non-T cells using a Pan T Cell Isolation Kit (Miltenyi Biotec, UK) following the manufacturer's instructions. Following T cell isolation, the non-T cell fraction was collected for the experiment by eluting in the absence of the magnetic field. In experiments where T cell activation was required, non-purified lymphocyte preparations were exposed to anti-mouse CD3e whereas purified T cell preparations were exposed to both anti-mouse CD3e and anti-mouse CD28.

Assessment of neuronal death

OSCs (13-15 DIV) were checked under bright field microscopy for well-defined cytoarchitecture prior to use for experiments. All drugs and lymphocytes ($1X10^6$ cells per ml) were mixed in fresh culture media and equilibrated at 37^0C/5% CO_2. Conditioned media was prepared by centrifuging (2000 rpm, 10 minutes) media containing lymphocytes which were incubated for 18 hours at 37^0C / 5% CO_2 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; 2µM) following exposure to kainic acid (KA) for one hour and incubated for 18 hours at 37^0C/5% CO_2. 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 37^0C/5% CO_2. Following treatment, OSCs were transferred to a microscope-mounted incubator chamber (37^0C; 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.[8] A concentration-response curve was generated using KA (3µM to 3mM) to determine maximum neuronal death.

All data are presented as mean±S.E.M. with the number of slices indicated in 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.

RESULTS

Lymphocytes are not toxic to OSCs

Lymphocytes have been suggested to be either protective or detrimental to the CNS depending on the CNS state so, we determined whether lymphocytes were neurotoxic to OSCs under our experimental conditions. Initially, OSCs were treated with increasing concentrations of KA (3µM – 3mM, one hour) to determine the maximum cell death. As per previous studies in our laboratory, KA induces neurotoxicity in a concentration-dependent manner with maximum cell death being observed at 3mM (Fig 1A and B).[8] Hence in all further experiments, OSCs were treated with kainate (KA, 300µM, one hour) as a positive control

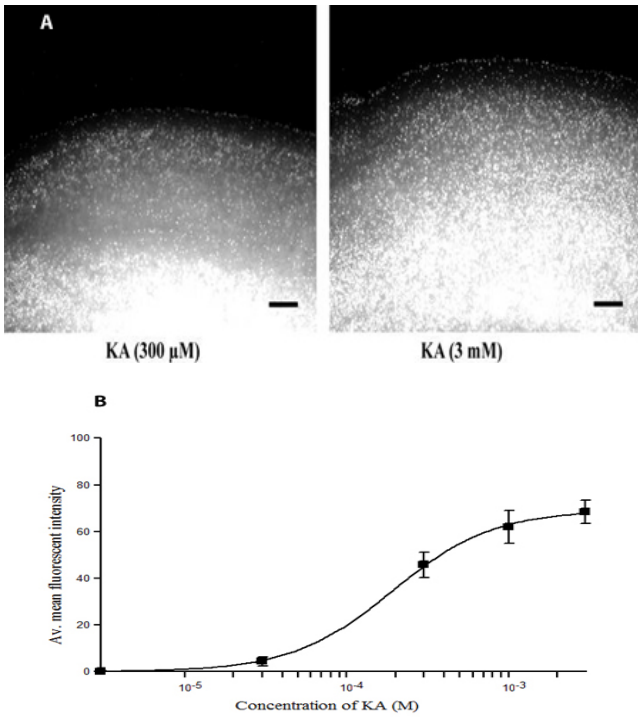

Figure 1. Kainic acid shows neurotoxicity in concentration-dependent manner. (A) Representative images showing propidium iodide fluorescence in 18hrs after 1hr exposure to concentrations of KA 300µM and KA 3mM respectively. (B) Graph showing concentration-dependent manner of various concentrations of kainic acid. All experiments were carried out in at least 4 separate animal preparations. Data presented as mean ± SEM. Scale bar: 200 µm.

to induce neuronal death, which was completely abolished in the presence of both the AMPA/KA antagonist, NBQX (20µM), and the NMDA antagonist, DL-AP5 (100µM; n≥4; $p<0.001$ compared to KA (300 µM) alone; Fig 2). In contrast to the effects of KA, mixed lymphocyte preparations, either in the absence (18hr, n=17) or presence (18hr, n=9)

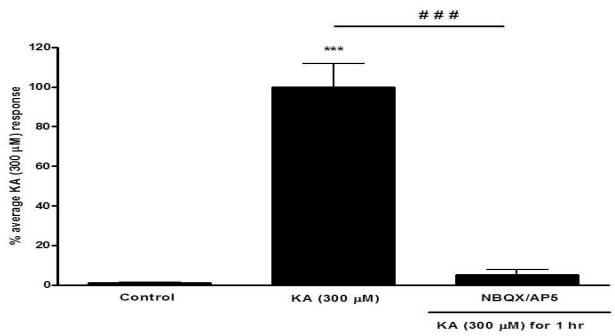

Figure 2. Bar chart showing inhibition of kainic acid-induced neurotoxicity by AMPA/KA antagonist NBQX and NMDA antagonist, DL-AP5. All experiments were carried out in at least 4 separate animal preparations. Data presented as mean ± SEM. ***p < 0.001 versus fresh culture media (control); ###p < 0.001 versus NBQX/AP-5 treated slice.

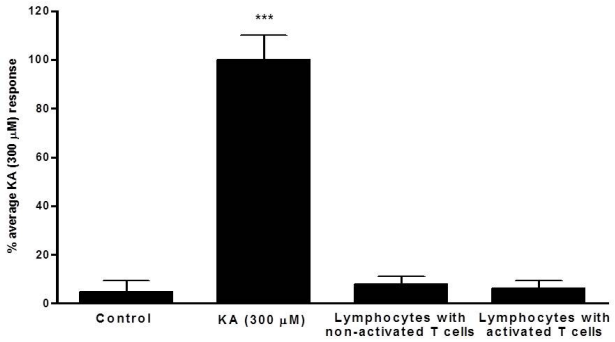

Figure 3. Bar chart revealing that lymphocytes themselves do not induce toxic effect to organotypic slice cultures. All experiments were carried out in at least 4 separate animal preparations. Data presented as mean ± SEM. ***p < 0.001 versus fresh culture media (control).

of activated T cells (antiCD3e antibodies mediated T cell activation), did not induce neurotoxicity in OSCs when exposed to lymphocytes for up to 18hr ($p>0.05$ compared to control, Fig 3).

Lymphocyte preparations exhibit neuroprotection against KA-induced cell death

Having established that lymphocyte preparations are not neurotoxic to OSCs, we investigated whether lymphocytes modulates KA-induced excitotoxicity in OSCs. A significant neuroprotection was observed when OSCs were incubated in lymphocytes for 18hr following exposure to KA (300µM, one hour). The lymphocyte-mediated neuroprotection was independent of T cell activation as KA-induced toxicity was reduced to 74.9±5.2% (n=48, $p<0.01$) of the 300µM KA control (Fig 4A) and 79.1±4.8% (n=50, $p<0.05$ compared to 300µM KA alone (Fig 4A) for preparations containing non-activated and activated T cells respectively.

T cells and B cells both exhibit neuroprotection against KA-induced cell death

We further confirmed that approximately 70% of the cells in our lymphocyte preparations were T cells (68.5± .8 %, n=16; Figure not shown) using fluorescence-activated cell

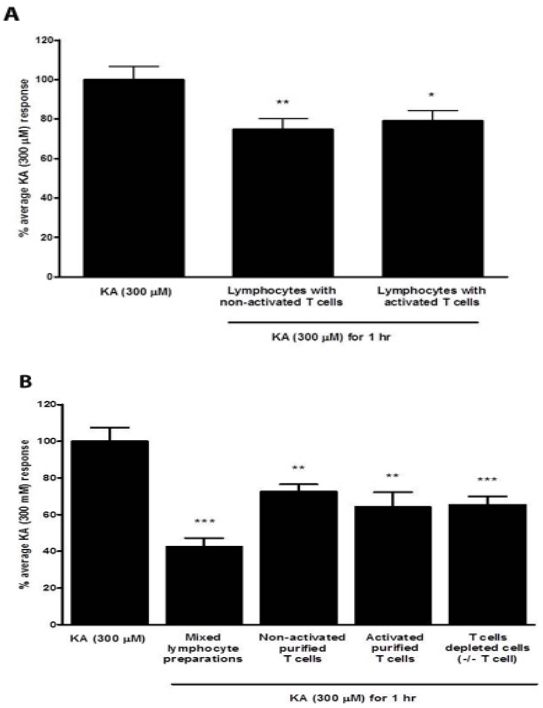

Figure 4. Lymphocytes are neuroprotective against kainic acid-induced neurotoxicity. (A) Bar chart showing that lymphocyte preparations containing either non-activated T cells or anti-CD3e activated T cells demonstrate neuroprotection against KA (300 µM, 1 hr). All experiments were carried out in at least 15 separate animal preparations. Data presented as mean ± SEM. *p < 0.05, **p < 0.01 versus KA (300 µM). (B) Bar chart showing neuroprotective effect of purified non-activated and purified activated T cells. All experiments were carried out in at least 6 different animal preparations. Data presented as mean ± SEM. *p < 0.05, ***p < 0.001 versus KA 300 µM.

sorting (FACS) analysis. Therefore, to investigate the role of isolated lymphocyte cell types in the observed protection against KA-induced neuronal death, experiments were performed using mixed lymphocyte preparations and these were compared to the effects observed with cell types purified from the same mixed lymphocyte preparation. In agreement with our initial findings, mixed lymphocyte preparations significantly reduced cell death in OSCs from 42.6±4.6 % (n=18, p<0.001) of the 300µM KA alone (Fig 4B). Similarly, T cells purified from the same preparation significantly reduced KA-induced neuronal death, an effect that was independent of T cell activation state with cell death being reduced to 72.5±3.8 % (n=16, p<0.001) of the 300µM KA control (Fig 4B) and 64.2±8.0 % (n=7, p<0.01) of the 300µM KA control (Fig 4B) for non-activated and activated purified T cells respectively. As mixed lymphocyte preparations contain other cell types in addition to T cells, we examined whether T cell depleted lymphocyte preparations could also affect KA-induced cell death. Indeed, the lymphocyte preparations depleted of T cells significantly reduced KA-induced cell death (65.4±4.4 % of the 300µM KA control; n=18, p<0.001 compared to 300µM KA alone, Fig 4B). FACS analysis revealed that B cells are the majority cell type in these T cell depleted preparations (69.9±2.1 %, n= 4, Figure not shown) with the remaining cell types presumably being made up of dendritic cells, macrophages and natural killer cells.

Soluble mediators and astrocytic activation underlie lymphocyte-induced neuroprotection

Having established that lymphocyte preparations are neuroprotective under our experimental conditions and both T cells and B cells contribute to this, we investigated the mechanisms underlying the observed neuroprotection. To determine whether soluble mediators underlie lymphocyte-mediated neuroprotection, conditioned media was taken from mixed lymphocyte preparations and experiments performed to determine whether conditioned media from mixed lymphocyte preparations affected KA-induced neurotoxicity. Cell death in OSCs maintained in conditioned media (18hr) following exposure to KA (300µM, one hour) was significantly reduced to 69.5±7.1% (n=11, p<0.001, Fig 5A) of the 300µM KA control using media conditioned in the presence of both lymphocytes and OSCs (Cond. Media-I) and to 60.8±3.2% (n=10, p<0.001) of the 300µM KA control (Fig 5A) using media conditioned using lymphocytes alone (Cond. Media-II) indicate that the soluble mediators responsible for the neuroprotective effects are solely produced by the lymphocytes. In contrast, the neuroprotective effect of conditioned media was abolished when the media was heated

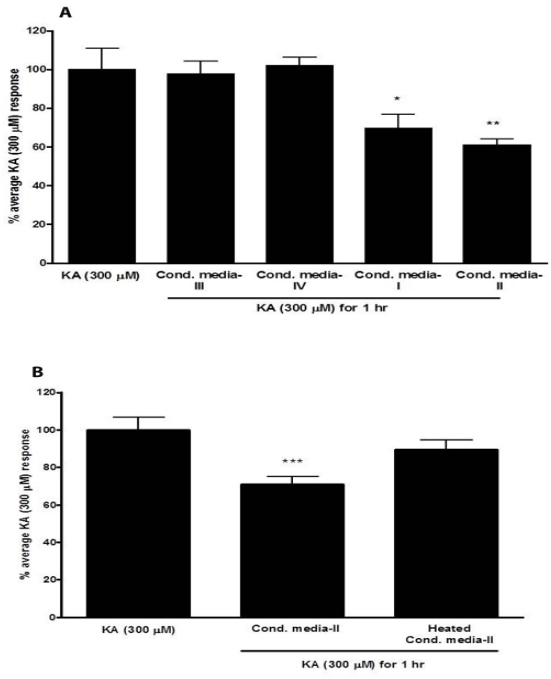

Figure 5. Soluble mediators are neuroprotective against KA-induced neurotoxicity. (A) Bar chart demonstrating that soluble mediators are released from lymphocytes and solely responsible for the observed neuroprotection. (B) Bar chart showing that soluble mediators from the conditioned media are proteinaeous. All experiments were carried out in at least 5 separate animal preparations. Data presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 versus KA 300 µM.

(87.2±5.0%, n=7, p>0.05 versus 300µM KA alone, Fig 5B). Furthermore, to confirm that lymphocytes were essential for the conditioned media-mediated neuroprotection, conditioned media from KA treated slices (97.6±6.5%, n=9, p>0.05 versus 300µM KA alone, Cond. Media-III) and conditioned media from primary hippocampal cultures (101.8±4.4%, n=9, p>0.05 versus 300µM KA alone, Cond. Media-IV) were taken. Both media were ineffective against KA-induced neurotoxicity. These data suggest that soluble mediators released from lymphocytes are responsible for the observed neuroprotection.

Further, to determine the type of cells within the OSCs responsible for the observed neuroprotection, we have investigated the role of astrocytes in our experimental condition using the glial metabolic inhibitor, fluoroacetate (FAc), which we have successfully used to investigate astrocyte function in previous studies.[8,9] Exposure of OSCs to FAc (10µM, three hour) did not affect KA-induced cell death (n=12, p>0.05 compared to 300µM KA alone, Fig 6) whereas neuroprotection elicited by conditioned media (78.8±4.1%, n=27, p<0.01 versus 300µM KA alone) was significantly reduced in FAc-treated OSCs (94.1±2.2%, n=21, p<0.05 versus 300µM KA + Conditioned media).

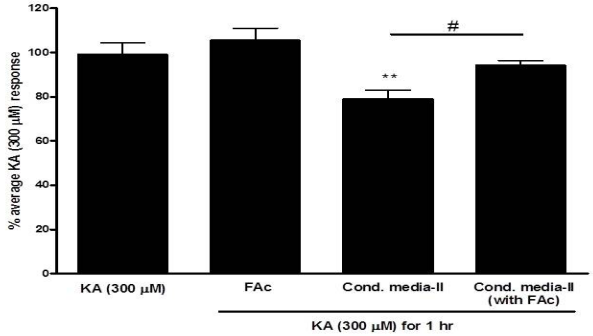

Figure 6. Bar chart showing that the observed neuroprotection is mediated via astrocyte as the glial toxin, FAc has blocked the neuroprotective effect of conditioned media. All experiments were carried out in at least 10 separate animal preparations. Data presented as mean ± SEM. #p < 0.05 versus FAc treated slice, **p < 0.01 versus 300 µM KA.

DISCUSSION

In the present study, we show that lymphocyte preparations are protective against KA-induced cell death under our experimental conditions and the observed neuroprotection is independent of T cell activation state. We suggest that the lymphocyte-mediated protection involves soluble mediators which act on astrocytes to induce neuroprotection.

The role of the immune system in modulating micro-environment of 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 primarily 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 neuropathology and a role for neuroinflammation has been implicated in numerous CNS diseases including Alzheimer's disease and Parkinson's disease.[10-12] However, studies also suggest that lymphocytes may have a neuroprotective role in the CNS when investigated using models of spinal cord injury, facial nerve axotomy and ischaemia.[13-16] Our findings are in agreement with a previous study which focused specifically on the consequence of exposing organotypic cultures to the specific T cell subtypes.[17] It revealed that both Th1 and Th2 cells were protective in a contact independent manner and the activation of these cell types led to increased neuroprotection. However in contrast to our study, this protection was against secondary damage initiated by explantation of tissue from living animal to the culture dish whereas our own study utilised established models of neurodegeneration.[7,18] Hence we have shown for the first time that lymphocytes are protective in established in vitro model of neurodegeneration. Furthermore, in our study, 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 with regard to T cell activation for example, but further investigation into this is beyond the scope of this study. As mentioned previously, T cells were neuroprotective which is in agreement with previous studies where the role of T cells has been investigated in in vivo models of neurodegeneration.[13,15,16] However, it should be noted that many of these studies were performed either in immune compromised or specific pathogen free animals and it could be argued that the observed effects may be due to the studies being carried out under such conditions.[19]

With regard to T cell subtypes and neuroprotection, studies have implicated Treg cells as being the major lymphocyte type involved in neuroprotection in experimental stoke and in experimental autoimmune encephalitis (EAE) models.[16,20] Neurons have been proposed to induce Treg cells from encephalitogenic T cells and these Treg cells function to suppress EAE.[21] In addition to Treg cells, Th2 cells have been suggested to play a role in neuroprotection but there are discrepancies as to whether this occurs in a contact-dependent or contact-independent manner.[17,22-24] Our findings support the suggestion that T cells can be neuroprotective however further investigation is required to establish the responsible T cell subtypes for these effects under our experimental conditions. Despite the majority of studies focussing on the role of T cells in CNS disorders, recent studies have also proposed a possible role for B cells. Studies have shown that the absence of B cells causes a significant increase in the infarct size of the experimental

stroke model whereas disease severity was increased in EAE models following B cell depletion.[20,25] Furthermore, adoptive transfer of B cells from glatiramer acetate-treated mice suppresses EAE compared to those injected with PBS or ovalbumin, indicating that the protective effects of glatiramer acetate against MS may be mediated via B cells.[26] In agreement with these studies, T cell depleted lymphocyte preparations, which contain approximately 70% B cells as determined by FACS analysis, were protective against KA-induced neurotoxicity thus adding further support for B cells playing a protective role against certain neurological disorders.

Having established that lymphocyte preparations including T cells depleted cells are neuroprotective and independent of activation state of T cells, we then sought to identify the underlying mechanisms. We confirmed that soluble mediators were responsible for the observed neuroprotection as conditioned media was protective against KA-induced neurotoxicity. Furthermore, as the conditioned media when heated loose the neuroprotective property, we conclude that proteins within the media underlie this neuroprotection. The ability of T cells and B cells to produce a number of chemokines, cytokines and neurotrophic factors is well established with strong evidence for neuroprotective mediators including interleukin (IL)-10 transforming growth factor (TGF)-β and brain-derived neurotrophic factor (BDNF).[15,19,23,27-31] Here, instead of determining the specific soluble mediators, we decided to investigate whether these soluble mediators interact with a specific cell type within OSCs to induce the neuroprotection. OSCs are made up of a heterologous population of cell types including astrocytes, microglia and neurons, hence we investigated whether inhibition of astrocytic activity would modulate the lymphocyte-mediated neuroprotection using already established techniques in our laboratory.[8,9] Inhibition of astrocytic activity using the glial toxin, FAc, abolishes lymphocyte-mediated neuroprotection against KA-induced neurotoxicity. 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 whereas it is now proposed that astrocytes can play a key role in modulating synaptic transmission and plasticity.[32-34] Furthermore, recent studies have shown the role of astrocytes in neurodegeneration associated with certain CNS disorders including Alzheimer's disease and Parkinson's disease.[32,35] However, there is a debate on 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. Under our experimental conditions, astrocytes have shown a role in the observed neuroprotection against KA-induced toxicity as shown by the loss of neuroprotection when astrocytic function is inhibited using the glial toxin, FAc. The ability of astrocytes to release neurotropic factors is thought to contribute to their role in neuroprotection as BDNF and glial derived neurotrophic factor (GDNF) known to be neuroprotective in models of neurodegenerative disorders for many years.[36,37] Recently, GDNF has been the subject of intense investigation regarding its potential therapeutic use in Parkinson's disease.[38,39] However, neurotrophins released by astrocytes 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 whereas under our experimental conditions, lymphocyte-mediated neuroprotection is contact-independent.[24] Considering this evidence, it is clear that lymphocytes are protective in KA-induced neurotoxicity and the soluble mediators involved may have direct actions in neuroprotection either directly or indirectly via astrocytes.

CONCLUSION

The present study demonstrates that lymphocytes are neuroprotective against KA-induced toxicity under our experimental conditions and this is independent of T cell activation state. We also propose that this neuroprotection is mediated indirectly by the action of soluble mediators on astrocytes. Our data indicate that lymphocytes may play multiple roles in CNS disorders which are in agreement with previous studies which suggest a neuroprotective role for lymphocytes in models of neuronal death. However, further investigation is required to find out the underlying mechanism of the observed neuroprotection for the possible therapeutic intervention in neurodegeneration of the CNS.

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