doi: 10.1111/j.1365-2990.2010.01122.x

Fusion between human mesenchymal stem cells and rodent cerebellar Purkinje cells

K. Kemp*, D. Gordon*, D. C. Wraith†, E. Mallam*, E. Hartfield‡, J. Uney‡, A. Wilkins* and N. Scolding*

**Multiple Sclerosis and Stem Cell Group, Institute of Clinical Neurosciences, Clinical Sciences North Bristol, †Department of Cellular and Molecular Medicine, and ‡Henry Wellcome Laboratories for Integrative Neuroscience and endocrinology, Dorothy Hodgkin Building, University of Bristol, Bristol, UK*

K. Kemp, D. Gordon, D. C. Wraith, E. Mallam, E. Hartfield, J. Uney, A. Wilkins and N. Scolding (2011)
Neuropathology and Applied Neurobiology **37**, 166–178

Fusion between human mesenchymal stem cells and rodent cerebellar Purkinje cells

Aims: We explored whether cellular fusion and heterokaryon formation between human and rodent cells in the cerebellum of mice occurs after intravenous injection of human bone marrow-derived mesenchymal stem cells (MSCs). The influence of central nervous system inflammation on this process was also assessed. In addition, we examined whether tumour necrosis factor (TNF)-alpha and interferon (IFN)-gamma, factors associated with inflammation, increase cellular fusion between human MSCs and rodent cerebellar neurons *in vitro*. **Methods and results:** Human MSCs were intravenously injected into mice with experimental autoimmune encephalomyelitis (EAE) and control mice. After 22 days, mouse Purkinje cells expressing human Golgi Zone were found within the Purkinje cell layer of the cerebellum, indicating that fusion and heterokaryon formation had occurred. The numbers of heterokaryons in the cerebellum were markedly increased in mice with EAE compared with control mice. Rodent cerebellar neuronal cells labelled with enhanced green fluorescent protein *in vitro* were co-cultured with human bone marrow-derived MSCs in the presence of TNF-alpha and/or IFN-gamma to determine their influence on fusion events. We found that fusion between MSCs and cerebellar neurons did occur *in vitro* and that the frequency of cellular fusion increased in the presence of TNF-alpha and/or IFN-gamma. **Conclusions:** We believe that this is the first paper to define fusion and heterokaryon formation between human MSCs and rodent cerebellar neurons *in vivo.* We have also demonstrated that fusion between these cell populations occurs *in vitro*. These findings indicate that MSCs may be potential therapeutic agents for cerebellar diseases, and other neuroinflammatory and neurodegenerative disorders.

Keywords: fusion, mesenchymal stem cells, Purkinje cells, stem cell, transplantation

Introduction

Stem cell therapies hold much therapeutic promise in a variety of neurodegenerative diseases. Specifically, human mesenchymal stem cell (MSC) transplantation has been shown to improve outcome in a variety of animal models of neurological disease including experimental autoimmune encephalomyelitis (EAE), stroke and spinal cord injury [1–4]. Pilot studies of MSC therapy have also commenced in stroke [5], multiple system atrophy [6] and in multiple sclerosis within our institute [7].

Mesenchymal stem cells can be easily isolated from the bone marrow and rapidly expanded *in vitro* thus lending to the transplantation of MSC being an attractive therapeutic tool for central nervous system (CNS) injury. A variety of mechanisms by which MSCs might exert therapeutic

Correspondence: Kevin Kemp, MS labs, 1st floor, Burden Centre, Frenchay Hospital, Bristol BS16 1JB, UK. Tel: +44 117 340 6723; Fax: +44 117 340 6655; E-mail: kevin.kemp@bristol.ac.uk

Disclosures: The authors indicate no potential conflicts of interest.

© 2011 The Authors
Neuropathology and Applied Neurobiology © 2011 British Neuropathological Society

effects in neurodegenerative disorders have been postulated, including replacement of lost cells by differentiation into functional neural tissue (which remains controversial); modulation of the immune system to prevent further neurodegeneration; or provision of trophic support for the diseased nervous system [8,9]. In addition to these much studied mechanisms of neuroprotection, recent reports have indicated that bone marrow-derived stem cells can fuse with differentiated cells in a range of tissues, including the brain. This may be a physiological phenomenon to introduce young nuclei or functional genes in aged or degenerating cells [10].

Within the CNS, fusion of bone marrow-derived stem cells is seen, but may be restricted to Purkinje cells within the cerebellum [11,12], and appears extremely infrequent under normal biological conditions [13,14]; thus its significance and physiological importance remains unclear. However, an important observation has been that inflammation not only promotes migration and infiltration of bone marrow-derived stem cells to sites of brain injury [15–17], but also increases the frequency of stem cell fusion [11,12]. This raises the tentative possibility that fusion could represent a means of stem cell mediated neuroprotection or rescue of highly differentiated cell types which cannot be replaced in adults [18,19], and that immunological and inflammatory factors, recruiting endogenous stem cells and stimulating fusion, could help limit the loss of structural neurons such as Purkinje cells [11]. An understanding of the mechanisms underlying cell fusion events between bone marrow derived stem cells and Purkinje cells might lead to techniques to manipulate these mechanisms and introduce functional 'donor' cell genes or engineer 'donor' cells to express factors that may increase Purkinje cell survival and survival of adjacent cerebellar cells in patients with cerebellar degeneration.

Chronic inflammation could cause an increase in spontaneous fusion events through increasing cytokine levels, by activating immune cells or by damaging the blood brain barrier leading to increased permeability [11,12]. We hypothesize that endogenous factors associated with inflammation, such as tumour necrosis factor (TNF)-alpha and interferon (IFN)-gamma, may also directly activate stem cells and Purkinje cells to promote fusion. To confirm this, we demonstrate an increase in mouse cerebellar Purkinje cells labelling for the human Golgi Zone within the Purkinje cell layer of the cerebellum after intravenous infusion of MSCs into mice with EAE, an inflammatory CNS condition characterized by raised levels of both TNF-alpha and IFN-gamma. In addition, we show that fusion between MSCs and cerebellar neurons *in vitro* is increased in the presence of TNF-alpha and/or IFN-gamma. These findings demonstrate that bone marrow-derived stem cells may integrate into the cerebellum, in a manner which is influenced by inflammatory mediators; and thus may be potential therapeutic agents for inflammatory disorders affecting the cerebellum, such as multiple sclerosis.

Materials and methods

Bone marrow harvest

Bone marrow samples were obtained by Orthopaedic surgeons at the Avon Orthopaedic Centre, Southmead Hospital, Bristol, UK, with informed written consent and hospital ethic committee approval. Bone marrow was taken at the time of total hip replacement surgery from the femoral shaft and placed into sterile 50 ml tubes containing 1000 IU heparin. Patients with a history of malignancy, immune disorders or rheumatoid arthritis were excluded from the study. Femoral shaft bone marrow donors were healthy apart from osteoarthritis, and were not receiving drugs known to be associated with myelosuppression or bone-marrow failure.

Establishment of mesenchymal culture

Femoral shaft marrow samples were broken up with a scalpel and washed with Dulbecco's Modified Eagles Medium (DMEM, Sigma-Aldrich, Gillingham, UK) until remaining material (bone) looked white at the bottom of the 50 ml tube. All washings were pipetted into a new 50 ml tube and kept for centrifugation. The suspension was centrifuged and resuspended in DMEM and overlaid onto an equal volume of Lymphoprep™ (Axis-Shield, Dundee, UK; density 1.077 ± 0.001 g/ml) and centrifuged at 600 g for 35 min at room temperature to separate the mononuclear cells (MNC) from neutrophils and red cells. The MNC layer was harvested and washed twice in DMEM.

MSC culture

Isolated MNCs were centrifuged and resuspended in MSC medium (consisting of DMEM with 10% foetal calf serum (FCS) selected for the growth of MSC (StemCell Technologies, London, UK) and 1% Penicillin and Streptomycin

© 2011 The Authors

(Sigma-Aldrich, Gillingham, UK). Vented flasks (25 cm^2) containing 10 ml of MSC medium were seeded with 1×10^7 cells for primary culture. Flasks were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and fed every week with MSC medium by half medium exchange to remove non-adherent hematopoietic cells until the adherent fibroblast-like MSCs reached approximately 70% confluence.

On reaching confluence the adherent cells were resuspended using 0.25% trypsin (Sigma-Aldrich, Gillingham, UK) and reseeded at 2.25×10^5 cells per (75 cm^2) flask into first passage. Cultures were then incubated, fed every week with MSC medium by half medium exchange, and again trypsinized, a cell count taken and reseeded at 2.25×10^5 cells per flask (75 cm^2).

MSC characterization

Immunophenotyping of surface markers was carried out using flow cytometry, according to previous reports [20], to ensure a homogenous population of MSC had been cultured. Cells were examined at third passage using anti-CD105, anti-CD45 (eBioscience, San Diego, CA, USA), anti-CD166, and with anti-CD44 (Serotec, Oxford, UK). Mesenchymal stem cells were induced into adipogenic, osteoblastic and chondrogenic differentiation by culturing MSC, at third passage, in NH Adipodiff medium, NH Osteodiff medium and NH Chondrodiff medium (Miltenyi Biotec, Woking, UK), respectively, according to the manufacturers instructions. Adipogenic differentiation was visualized by the accumulation of lipid-containing vacuoles which stain red with oil red O and using immunofluorescent detection by labelling with anti-lipoprotein lipase (Abcam, Cambridge, UK). Osteogenic differentiation was visualized morphologically and also by the presence of high levels of alkaline phosphatase stained with NBT substrate and using immunofluorescent detection by labelling with anti-Alkaline phosphatase (Abcam, Cambridge, UK). Finally chondrogenic differentiation was characterized by Alcian blue staining and the production of the extracellular matrix proteoglycan aggrecan, visualized using immunofluorescent detection by labelling of aggrecan using a mouse anti-human aggrecan (4F4) antibody (Santa Cruz Biotechnology, Heidelberg, Germany).

MSC infusion into Naïve and EAE mice

All animal experiments were performed with institutional (University of Bristol) and British Home Office approval. C57/Bl6 mice were immunized by subcutaneous injection of complete Freund's adjuvant containing 200 ng myelin oligodendrocyte glycoprotein (MOG) peptide (35–55) at the base of the tail and intraperitoneal injection of 200 ng pertussis toxin per mouse. This was repeated 48 h later [21]. Six days later, mice were injected with either 1×10^6 human mesenchymal stem cells (3rd–6th passage, derived from one of the four separate donors used) or phosphate-buffered saline (PBS) (as a sham), via the intra-venous route. Mice were divided into four experimental groups: two groups were inoculated to induce EAE, and of these, one group was treated subsequently with (sham) PBS, one with normal human MSCs; of the remaining two (non-EAE) groups, one received (sham) PBS, the other with normal human MSCs. On Day 28, mice were sacrificed and brains were extracted for analysis. CNS tissue was fixed in 4% paraformaldehyde solution and 15 μm sections were cut and examined.

Transplantation of MSCs were performed without immunosuppressive anti-rejection therapy as MSC have demonstrated that they are able to evade the allogeneic immune system following intravenous infusion [22–25]. In addition, animal models in which allogeneic major histocompatibility complex mismatched MSC were infused or implanted have shown that MSC have been well tolerated and escape immune recognition in situations where they should be recognized by alloreactive T cells [26–29].

Analysis of cerebellar sections from naïve and EAE mice

Sagittally cut cerebellar sections were immunocytochemically labelled with Mouse monoclonal anti-human Golgi Zone (1:500) (Millipore, Watford, UK) and Rabbit anti-Calbindin-D28K (1:500) (Sigma-Aldrich, Gillingham, UK). Immunolabelled sections of the cerebellum were examined and numbers of human Golgi Zone/Calbindin-D28K positive cells from naïve and EAE mice were determined.

Neuronal cell culture

Neuronal cultures were prepared from the cerebella of E18 rat embryos. In brief, the pregnant female (time-mated), was sacrificed and embryos removed. The cerebella were dissected out and the meninges removed. Following enzymatic and mechanical dissociation, cells were counted and plated onto poly-L-lysine coated 13 mm coverslips at 250 000 cells/coverslip and cultured in

© 2011 The Authors

Dulbecco's modified eagles medium supplemented with 2% B27 (Gibco, Paisley, UK) and 1% Penicillin and Streptomycin. After 5 days *in vitro* culture, >95% of cells were positive for the neuronal marker βIII-tubulin, and within this population 20.1% ± 2.3 ($n = 3$) were Purkinje cells (identified by Calbindin D28K staining, a calcium binding protein that is expressed by Purkinje cells in the cerebellum). The remaining cells (<2.5%) were positive for the astrocyte and oligodendrocyte markers [glial fibrillary acidic protein (GFAP) and galactocerebroside (Gal-C), respectively] [30].

Enhanced green fluorescent protein (EGFP) transduction of E18 rat neuronal cultures

At 5 days *in vitro* culture, cerebellar neuronal cells were transduced (multiplicity of infection = 2) with a third generation lentivirus expressing EGFP. Briefly, all media were removed from the neuronal cultures and replaced with 0.5 ml of Dulbecco's modified eagles medium supplemented with 2% B27 (Gibco, Paisley, UK), 1% Penicillin and Streptomycin and the lentivirus. Plates were then incubated at 37°C in a humidified atmosphere containing 5% CO_2 for a further 5 days prior to use within *in vitro* culture experiments; >80% of cerebellar neuronal cells were positive for EGFP after 5 days in culture.

E18 rat neuronal cell/MSC co-culture and cellular fusion identification

At 10 days *in vitro* culture, EGFP-transduced rat cerebellar neuronal cells were washed extensively with DMEM and co-cultured with human-derived MSC at 5000 cells/well, in Dulbecco's modified eagles medium supplemented with 2% B27 (Gibco, Paisley, UK) and 1% Penicillin and Streptomycin, with the addition of 5 ng/ml of human TNF-alpha and/or IFN-gamma (PeproTech, Rocky Hill, NJ, USA). Cells were fed by half medium exchange every 3 days and after a further 15 days culture all cells were resuspended using 0.25% trypsin and plated onto poly-L-lysine coated 13 mm coverslips for 24 h. Cells were then immunocytochemically stained with mouse monoclonal anti-human Golgi Zone (1:500). Neuronal/MSC fused cells were identified as being EGFP/human Golgi Zone double positive cells. Cultures were analysed by cell counting of five random fields from at least three coverslips per experimental condition using a LEICA DM6000B microscope (×40) (Leica Microsystems, Milton Keynes, UK).

Statistical analysis

Counting data was analysed using non-parametric tests (Kruskal-Wallis with *post hoc* Dunn's-testing between groups). Paired *t*-tests were used for analysis of normally distributed data. Values are expressed as the mean ± SEM from at least three independent experiments, taking $P < 0.05$ to represent statistical significance.

Results

Characterization of MSC cultures

Cells harvested from femoral shaft marrows displayed typical characteristics of MSCs in culture. Mononuclear cell cultures derived from femoral shaft bone marrows produced confluent adherent layers of elongated fibroblast-like cells in mesenchymal culture conditions. Mesenchymal stem cells were characterized by their immunophenotype and differentiation potential at third passage of culture. Using flow cytometric analyses, MSCs were found to be uniformly positive for the mesenchymal markers CD105, CD166, CD44, but negative for CD45 which is consistent with the known MSC phenotype and excludes contamination of cultures with haemopoietic cells [31]. In addition, MSCs were successfully induced to differentiate towards osteogenic, adipogenic and chondrogenic lineages using the methods described (Figure 1).

EAE increases numbers of human cells within the cerebellum of mice treated with human MSCs

The EAE status of the mice was confirmed using clinical EAE scores recorded using a standard scoring system (0 – Normal; 1 – Tail flaccidity or hind limb weakness; 2 – Partial hind limb paralysis; 3 – Complete hind limb paralysis, spastic paresis, impaired righting reflex; 4 – Complete hind and fore limb paralysis; 5 – Dead) [4]. In addition, both solochrome cyanine staining and immunofluorescent staining with an anti-myelin basic protein antibody was used to identify EAE brain and spinal cord lesions within the EAE induced mice (data not shown). No lesions were observed in the naïve mouse population, and all naïve mice presented normal clinical scores (data not shown).

A human specific anti-human Golgi Zone monoclonal antibody was used to detect human-derived cells post intra-venous MSC infusion in mice. The human Golgi

© 2011 The Authors

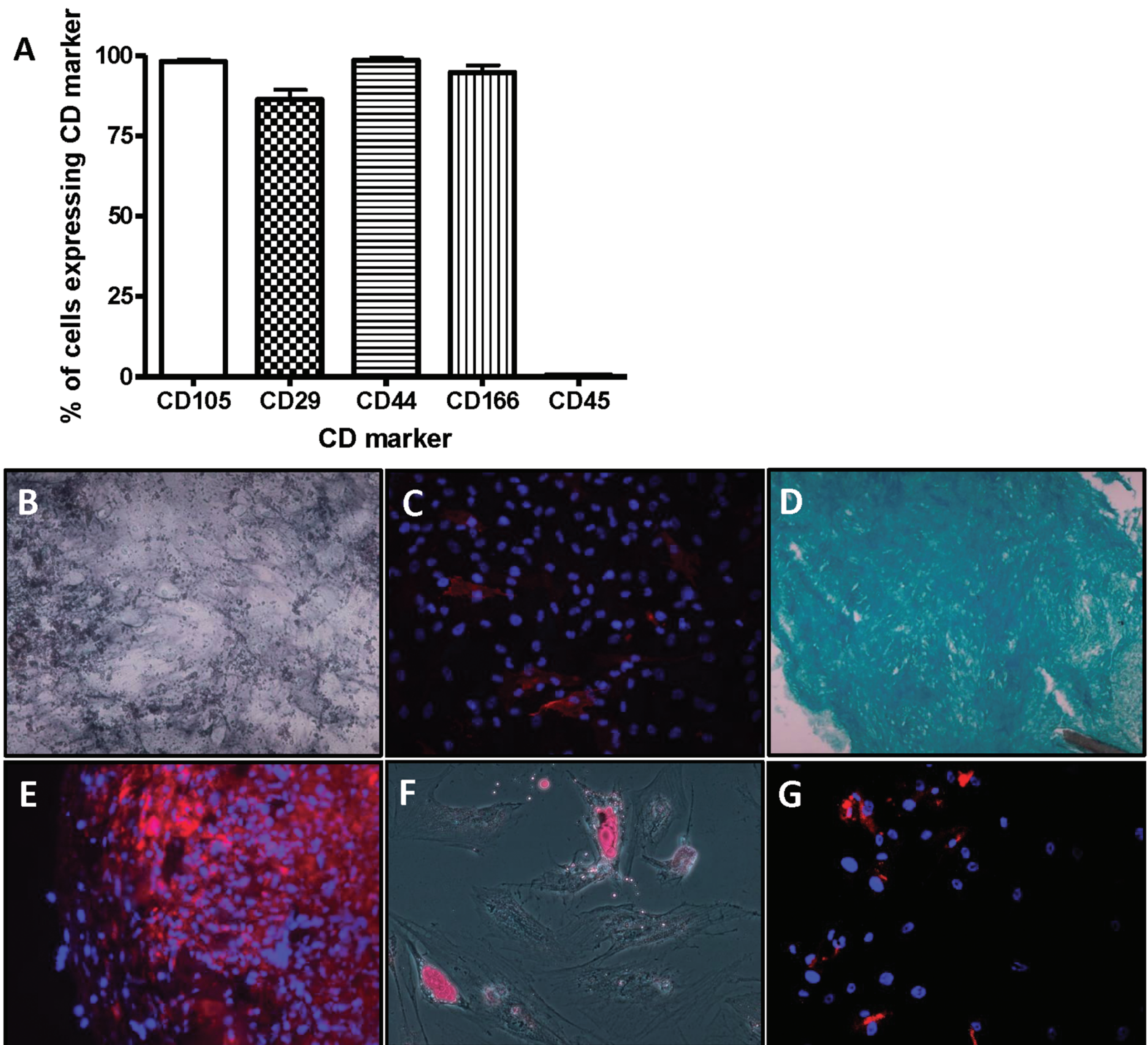

Figure 1. Mesenchymal stem cell cultures display a typical MSC phenotype and can be induced to differentiate towards osteogenic, adipogenic and chondrogenic lineages. (**A**) Flow cytometric analysis of CD29, CD105, CD166, CD44 and CD45 expression by mesenchymal stem cell (MSC) cultures at third passage ($n = 4$). Images depicting MSC cultures, at third passage, differentiated down osteogenic, adipogenic and chondrogenic lineages. Osteogenic differentiation was visualized by the presence of high levels of alkaline phosphatase (**B**) and using immunofluorescent detection of Alkaline phosphatase (red)/nuclei (blue) (**C**). Chondrogenic differentiation was characterized by Alcian blue staining (**D**) and the immunofluorescent detection of aggrecan (red)/nuclei (blue) (**E**). Adipogenic differentiation was visualized by the accumulation of lipid-containing vacuoles which stain red with oil red O (**F**) and using immunofluorescent detection of lipoprotein lipase (red)/nuclei (blue) (**G**).

Zone antibody specificity to cells of human origin was tested on both human derived MSC and rodent cerebellar neuronal cultures (Figure 2). Using this antibody, Golgi staining was completely absent within cerebellar cells of rodent origin. However, 100% of MSCs were positive for the Golgi Zone marker (Figure 2). Human Golgi Zone staining was also absent when tested on cerebellar tissue sections derived from control mice that had not received an infusion of MSC (data not shown).

Analysis of cerebellar sections from both naïve and EAE mice infusion revealed the presence of mouse Purkinje cells, expressing Calbindin-D28K, co-expressing human

© 2011 The Authors

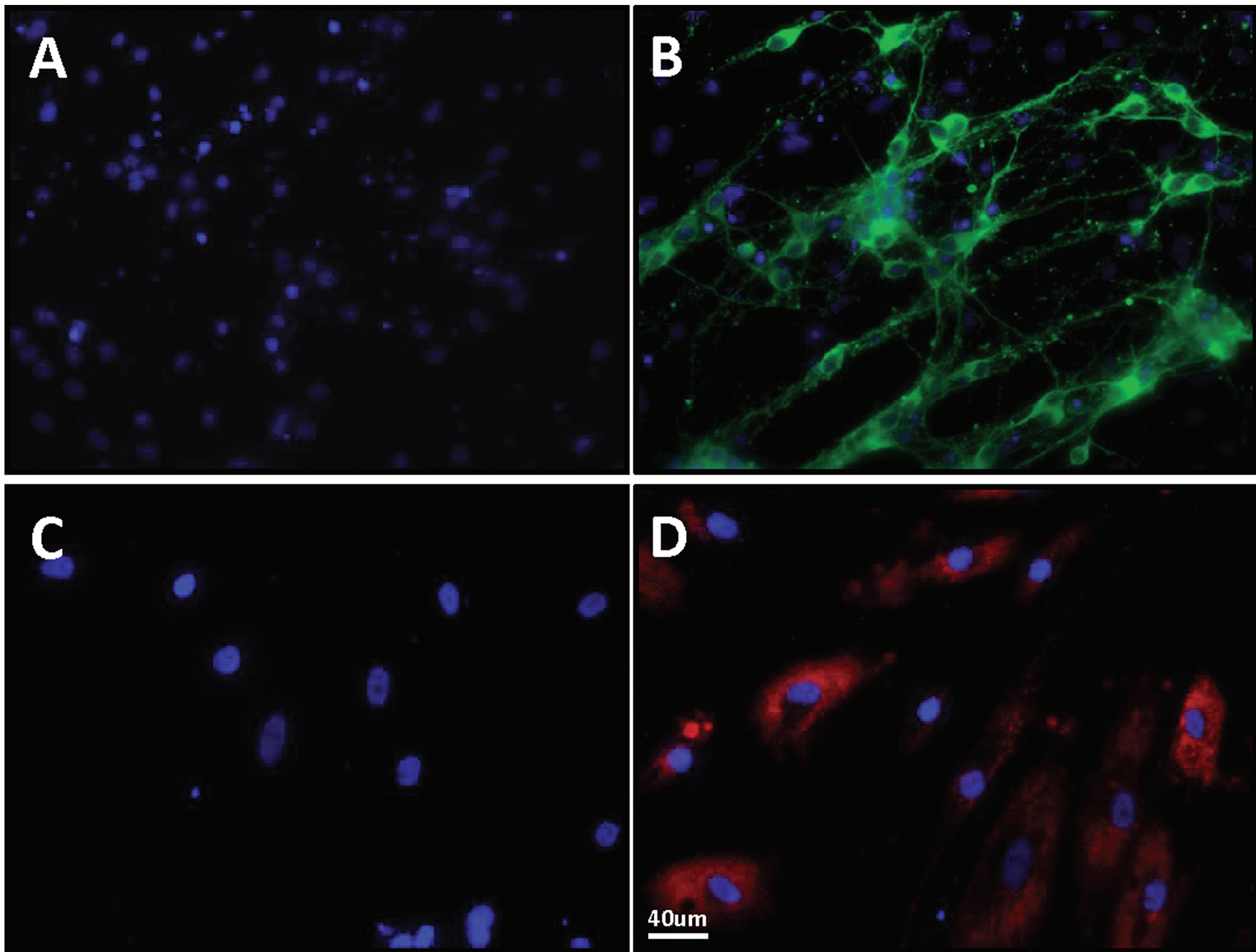

Figure 2. Anti-human Golgi Zone binds specifically to human mesenchymal stem cell (MSC) and not rodent cells. Images are of rat E18 cerebellar neuronal (**A**/**B**) and human MSC (**C**/**D**) cultures co-labelled with human Golgi Zone (red), βIII tubulin (green) and Hoescht nuclear stain (blue) (**A&C** are images of respective negative controls using no primary antibodies).

Golgi Zone within the Purkinje cell layer of the cerebellum of animals 22 days post intra-venous injection of MSC (Figure 3).

To investigate whether the induction of EAE increases the levels of cellular fusion and heterokaryon formation within the mouse cerebellum, the numbers of human Golgi Zone/Calbindin-D28K positive mouse Purkinje cells from naïve and EAE mice were determined. Immunolabelled sagittal sections of the cerebellum were examined and numbers of human Golgi/Calbindin-D28K double positive cells and total numbers of Calbindin-D28K positive cells were determined [10 complete sagittal sections of the cerebellum (Figure 3**D**) from five animals/treatment were investigated]. Within naïve animals a frequency of 0.147 ± 0.046% of Purkinje cells were positive for human Golgi Zone, compared with the significantly higher level of 1.454 ± 0.629% evident within the EAE group ($P < 0.01$) (Figure 4**A**). There were no differences in the number of Purkinje cells per random field counted within the cerebellum of EAE versus naïve animals 22 days post infusion with MSC (Figure 4**B**).

Characterization of cerebellar neuronal cultures

E18 cerebellar neuronal cells were cultured in serum-free B27-supplement for 5 days after plating. At this point neurons (identified by β-tubulin III staining) represented 97.9% ± 1.2 ($n = 3$) of the total cells, and within this population 20.1% ± 2.3 ($n = 8$) expressed the Purkinje cell marker Calbindin D28K [32]. The remaining cells were predominantly GFAP expressing astrocytes and Gal-C expressing oligodendrocytes.

© 2011 The Authors

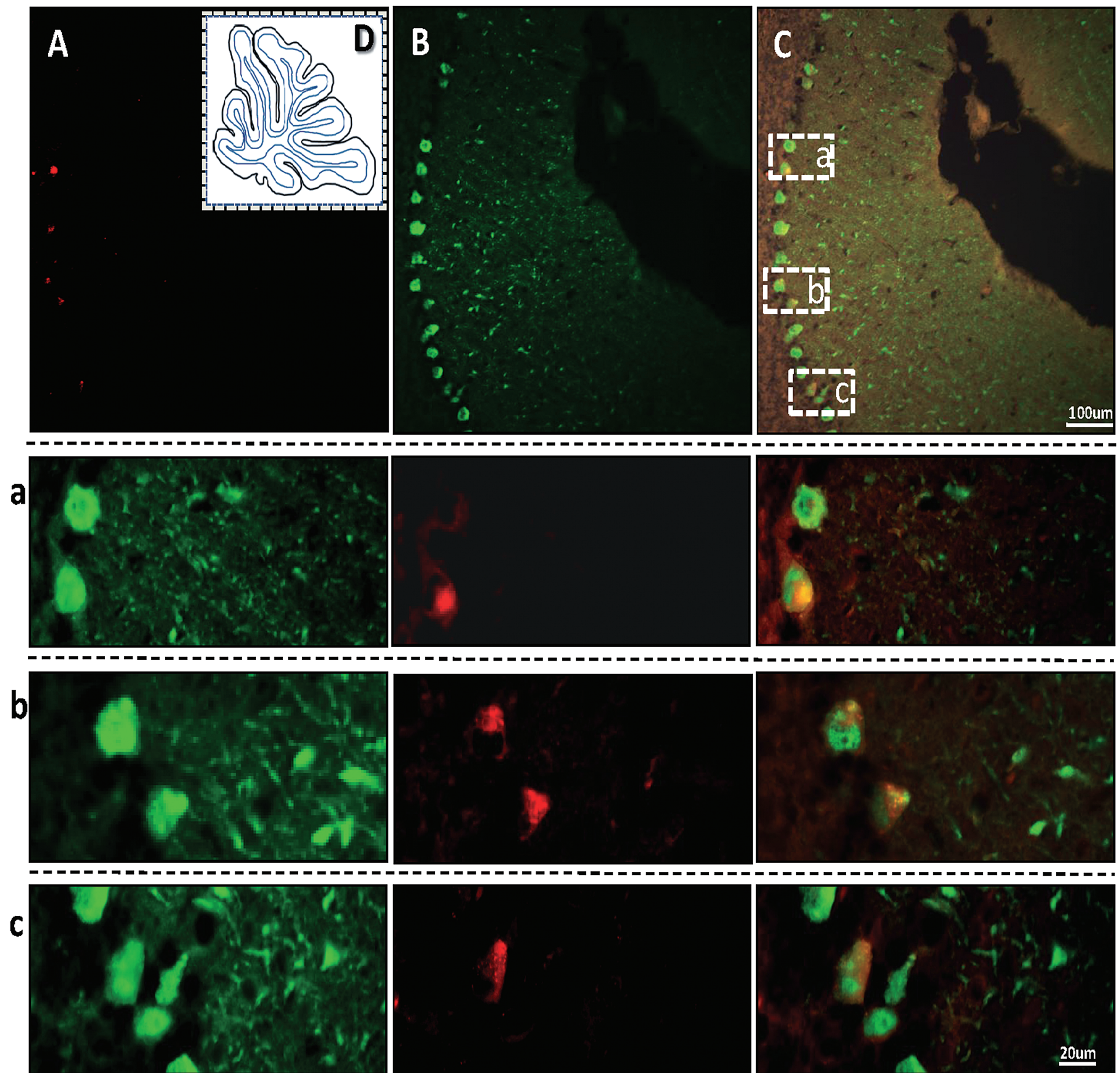

Figure 3. Mouse Purkinje cells stain for human Golgi Zone within the cerebellum of Naïve/experimental autoimmune encephalomyelitis (EAE)-mice treated with intravenous human mesenchymal stem cells (MSCs). Immunofluorescence photographs (a), (b) and (c) refer to dashed areas a, b, c in image (**C**). A cerebellum section from an EAE-mouse treated with intra-venous human MSCs immunologically labelled with human Golgi Zone (**A**) (red) and Calbindin-D28K (**B**) (green) and, merged images (**C**). (**D**) A diagram of a typical and complete area of a sagittally sectioned mouse cerebellum in which the presence of human MSCs was investigated and numerated.

Induction of MSC/cerebellar neuronal cell fusion *in vitro*

To investigate MSC/cerebellar neuronal cell fusion and test whether inflammatory cytokines TNF-alpha and IFN-gamma induce a higher frequency of fusion, human MSCs were co-cultured with EGFP-expressing rat cerebellar cells *in vitro*.

After 15 days co-culture, fused cells were identified using immunofluorescence microscopy as EGFP^{+ve}/human Golgi Zone^{+ve} cells. Fused cells were found in all MSC/neuronal cell co-cultures (Figure 5). The majority of

© 2011 The Authors

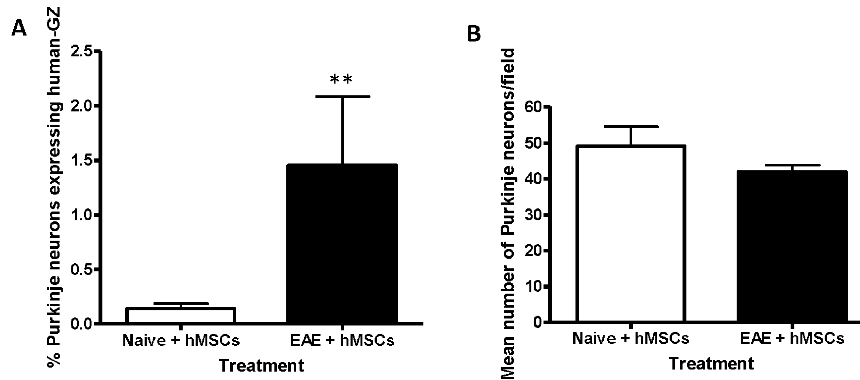

Figure 4. An increase in mouse Purkinje cells expressing human Golgi Zone in experimental autoimmune encephalomyelitis (EAE) vs. naïve mice treated with intra-venous human MSCs. (**A**) Quantification of Purkinje cells co-expressing human Golgi Zone in naïve mice ($n = 5$) and EAE mice ($n = 5$) 22 days post MSC infusion (**$P < 0.01$). (**B**) Quantification of the mean number of Purkinje cells per random field within the cerebellum of EAE and naïve mice ($n = 5$) 22 days post MSC infusion.

fused cells were shown to be bi-nucleate, although in a proportion of double positive cells only one nucleus was identifiable. No differences were seen in MSC numbers after the culture period between conditions (Figure 6**B**); however, there was a significant increase in neuronal survival evident when exposing neuronal/MSC cultures to both TNF-alpha and IFN-gamma when compared with base media alone (Figure 6**A**). The frequency of fusion events (demonstrated by double labelling with EGFP and human Golgi Zone) between the two cell populations was significantly increased by the addition of TNF-alpha and/or IFN-gamma to co-cultures when compared with the absence of cytokines (Figure 6**C**). Given the increase in neuronal numbers seen in co-cultures exposed to TNF-alpha and IFN-gamma, we calculated the percentage of EGFP positive cells expressing human Golgi Zone, and found that TNF-alpha and IFN-gamma increased the proportion of double labelled cells, indicating that this was not merely a function of increasing neuronal numbers (Figure 6**D**).

Discussion

Here we have performed a series of experiments showing that following intravenous infusion into normal healthy mice, human bone marrow-derived mesenchymal stem cells migrate to the cerebellum and fuse with host Purkinje cells. We have also demonstrated that in EAE there is a greatly increased frequency (up to 10-fold) of fusion events and heterokaryon formation between human MSCs and rodent Purkinje cells. To our knowledge, this is the first demonstration of the ability of human cells to fuse with cerebellar neurons in the context of EAE. This may have significant implications for therapies for human inflammatory diseases of the CNS. *In vitro* studies have allowed us to explore the direct effect of inflammatory mediators on heterokaryon formation. We have demonstrated that fusion between MSCs and cerebellar neurons can occur spontaneously *in vitro*, but with a markedly higher incidence in the presence of the inflammatory mediators TNF-alpha and IFN-gamma.

Previous reports have demonstrated fusion between rodent haematopoietic stem cells and rodent Purkinje cells, with increased levels occurring in the context of CNS or systemic inflammation [11]. The comparable increase in fusion was postulated largely to be due to the combination of CNS inflammation and stress to Purkinje neurons in the cerebellum causing a microenvironment conducive to cerebellar fusion and heterokaryon formation but the precise mechanisms have not been explored [11]. Increased migration of stem cells into the cerebellum as a consequence of inflammation could contribute; and the effects of inflammatory processes could conceivably include a direct action on Purkinje cells and stem cells. Alternatively, increased fusion could be mediated as a secondary effect of infiltrating cells and molecules on local cell populations, including microglia and/or astrocytes, both of which are well known to respond to such environmental changes.

Weimann *et al*. found that bone marrow-derived stem cells contributed to Purkinje neurons in the brains of adult women who had received bone marrow from male

© 2011 The Authors

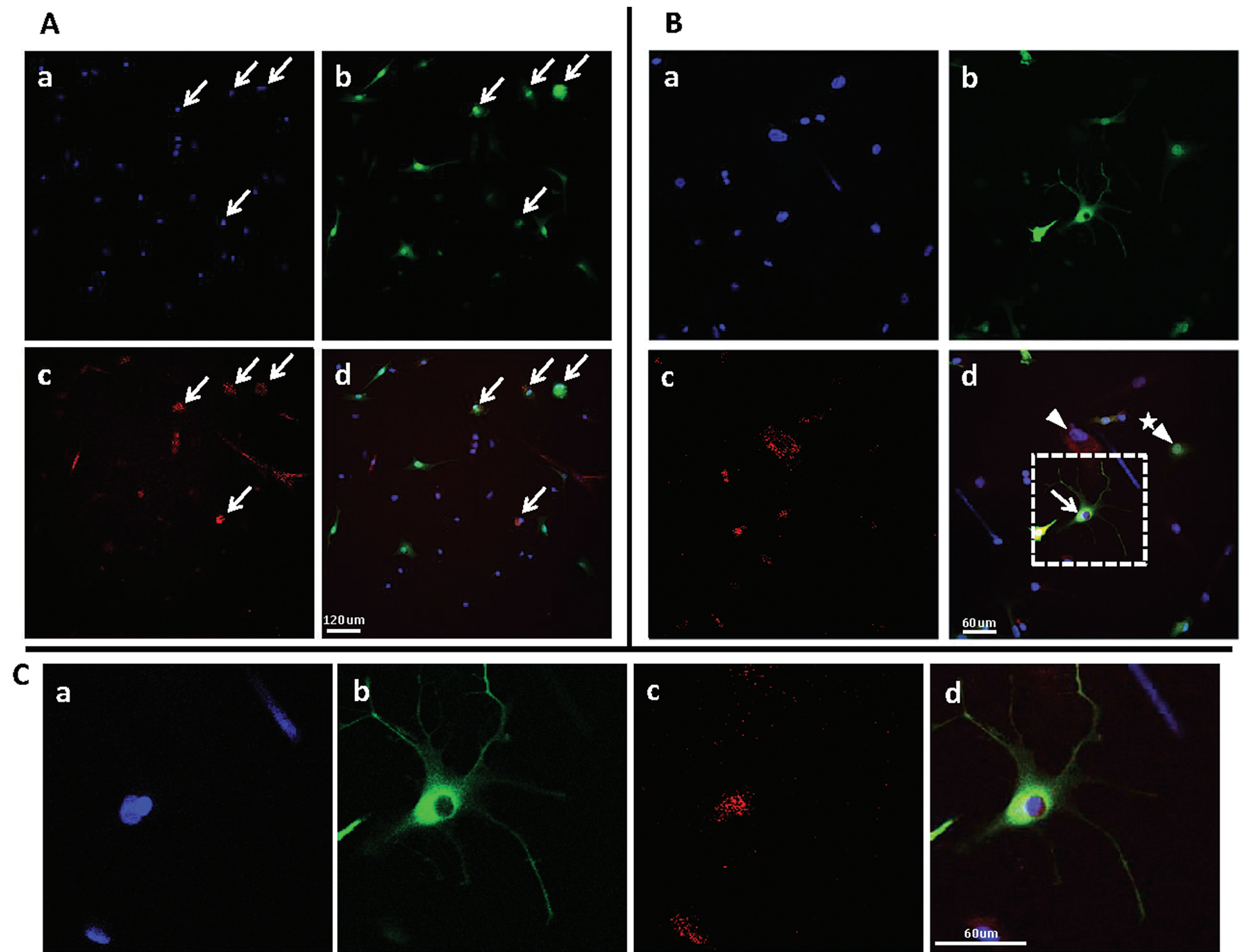

Figure 5. Human mesenchymal stem cells (MSCs) fuse with rat cerebellar enhanced green fluorescent protein (EGFP)-transduced neurons *in vitro*. Confocal images are of rat EGFP-transduced E18 cerebellar neuronal/human MSC co-cultures immunologically labelled with Hoescht nuclear stain (blue; a), EGFP (green; b), human Golgi Zone (red; c) and merged image (d). (**A**) Low power figure; arrows indicate dual stained EGFP/human Golgi Zone (red/green) cells; (**B**) high power figure; arrow head (human Golgi Zone positive/EGFP negative MSC), arrow head and star (human Golgi Zone negative/EGFP positive neuron), arrow (human Golgi Zone positive/EGFP positive fused MSC/neuronal cell). (**C**) An enlarged area of the dashed box in (**B**) showing a bi-nucleated human Golgi Zone positive/EGFP positive fused MSC/neuronal cell.

donors for the treatment of haematological malignancies; though they considered fusion an unlikely mechanism [13]. Subsequently, a number of groups transplanted green fluorescent protein-expressing stem cells into lethally irradiated/non-irradiated mice demonstrating the transplanted cells contribute to some of the Purkinje neurons in the cerebellum, where they can remain for months post infusion [10–12,14,33]. Importantly, Bae *et al*. (2007) demonstrated that bone marrow-derived MSC/Purkinje neuron fusion-like events develop into electrically active neurons with functional synaptic formation in the cerebellum of mice with neurodegeneration. Thus, MSCs may be able to integrate into the CNS and contribute to the essential properties of mature neurons [34].

Here, we have shown that human bone marrow-derived MSCs also exhibit this potentially reparative action, fusing with Purkinje cells in the rodent cerebellum *in vivo;* and these fusion events are increased in the neuroinflammatory environment of EAE, with no apparent loss in Purkinje cell numbers. It can therefore be hypothesized that endogenous factors associated with the physiological mechanisms of EAE, causing CNS inflammation, demyelination and neuronal/axonal degeneration, promote increased cell fusion between

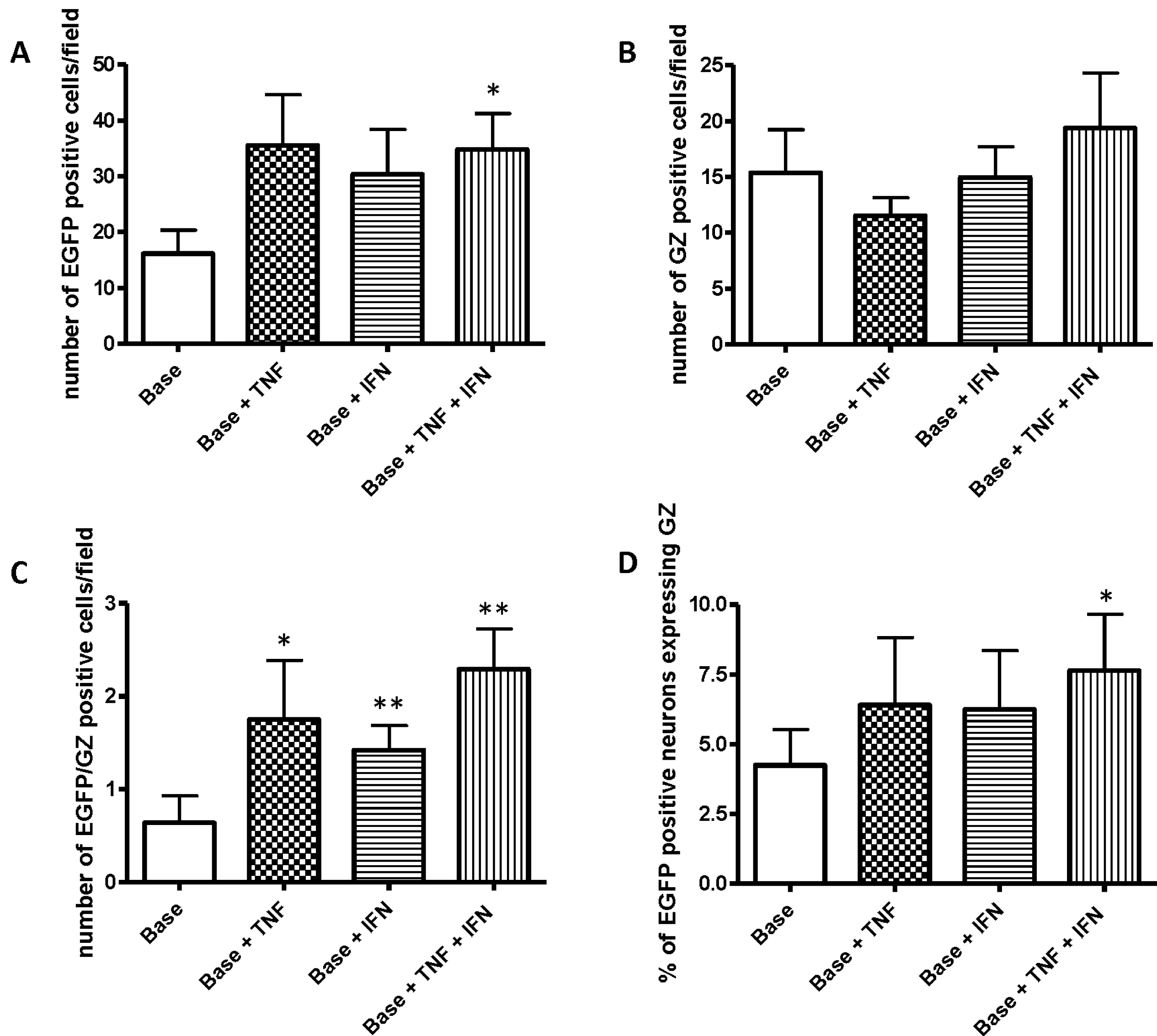

Figure 6. The effect of imflammatory cytokines tumour necrosis factor (TNF)-alpha/interferon (IFN)-gamma on human mesenchymal stem cell (MSC)/rat cerebellar neuronal fusion *in vitro* (*$P < 0.05$, **$P < 0.01$, $n = 4$). Co-cultured in base medium (Base); TNF (TNF-alpha 5 ng/ml), IFN (IFN-gamma 5 ng/ml) or TNF-alpha and IFN-gamma (5 ng/ml). (**A**) The number of enhanced green fluorescent protein (EGFP) positive neurons per field, (**B**) the number of Golgi Zone (GZ) positve MSCs per field, (**C**) the number of EGFP/GZ positive fused cells per field and (**D**) the percentage of EGFP positive neurons co-expressing human GZ for each experimental condition.

bone marrow-derived stem cells and Purkinje neurons within the cerebellum.

Although inflammation is not necessary to induce fusion between bone marrow-derived stem cells and Purkinje cells, fusion only occurs at very low levels under normal physiological circumstances [13,14]. Thus, inflammation following tissue damage seems to play an important role in heterokaryon formation. Recent studies indicate that increased fusion events are partly the result of inflammation-induced migration and infiltration of cells, linked to increased permeability of the blood-brain barrier [11,12]. While these processes appear to have an important role, Bae *et al*. (2005) have demonstrated that the cerebellar pathology associated with a non-inflammatory condition, murine Niemann-Pick disease type-C, augments the ability of bone marrow-derived MSCs to fuse with Purkinje neurons when transplanted directly into the cerebellum [35]. The results suggest that

© 2011 The Authors

increased fusion events may be, in part, independent of cell migration and blood brain barrier permeability, and that MSCs and/or Purkinje neurons respond to factors derived from the neurodegenerative microenvironment leading to increases in fusion events.

In EAE and other central nervous system inflammatory disorders, both IFN-gamma and TNF-alpha are up-regulated and are critically involved in the initiation and amplification of the local immune response in the CNS [36]. Cytokine levels increase in the murine CNS following induction of EAE by recombinant human MOG [37]. TNF-alpha and IFN-gamma secreting cells are present in the CNS of mice prior to clinical onset of MOG induced EAE symptoms, levels then rise around day 7, peaking at days 15–20 after immunization, followed by a decline during remission [38]. We therefore investigated whether MSC/neuronal cell fusion occurs *in vitro* and if these events are increased in response to the pro-inflammatory cytokines TNF-alpha and IFN-gamma. We have shown that fusion between MSCs and cerebellar neurons occurs *in vitro*, with an increase in cellular fusion in the presence of TNF-alpha and/or IFN-gamma. MSCs are known to express receptors for both TNF-alpha and IFN-gamma and signalling through these receptors can alter MSC gene expression profiles [39].It is therefore possible that MSC activation by TNF-alpha and IFN-gamma may represent at least one molecular mechanism by which MSCs are encouraged to fuse with Purkinje neurons. It is equally possible that these cytokines are acting through Purkinje neurons themselves, rendering neurons more susceptible to fusion events. It is interesting that an increase in neuronal survival was evident in the presence of both MSCs and TNF-alpha/IFN-gamma. It may be that this increase in survival is due to the protective nature of cellular fusion on neuronal cells. Alternatively, MSCs exposed to TNF-alpha/IFN-gamma may promote neuronal survival via other mechanisms. It is highly likely that cross-talk between the two populations, both *in vitro* and *in vivo*, is highly complex and warrants further study. We have, however, demonstrated an increase in the percentage of EGFP-expressing neurons co-expressing human Golgi Zone after cytokine exposure, and this, coupled with *in vivo* data showing increases in numbers of Purkinje cells also expressing human Golgi Zone after EAE induction, without any apparent loss in Purkinje cell numbers present within the cerebellum, indicates that inflammatory mediators have a direct and positive influence on cellular fusion between human MSCs and neuronal cells.

The biological relevance of cell fusion, either *in vitro* or *in vivo*, is, as yet, unclear, but may represent a physiological phenomenon to introduce young nuclei or functional genes in aged or degenerating cells [10]. The reasons why only Purkinje neurons, out of all of the subtypes of neurons in the CNS, appear to have the ability to fuse with bone marrow derived cells is of much interest. It may be that Purkinje cells have an embryological propensity to polyploidy and indeed this phenomenon has been documented in rodent Purkinje cells in other studies [40,41]. Furthermore, the observation that chronic inflammation promotes migration and infiltration of bone marrow-derived stem cells to the site of brain injury suggests a mechanism by which the body targets sites for neural repair [15–17]. We have shown that inflammatory mediators have the ability to increase the frequency of MSC fusion with neuronal cells *in vitro* along with increasing the frequency of human MSCs fusing with mouse Purkinje cells in the cerebellum *in vivo*. This strongly supports the notion that immunological factors involved in the inflammatory process have a role in limiting the loss of structural neurons, such as Purkinje neurons, which cannot be replaced in adult life [11]. Purkinje cell axons are the sole outputs from the cerebellar cortex where they make large numbers of contacts with several different cell types; they are therefore critical for normal cerebellar function [42]. Thus, it could be postulated that any preservation in Purkinje cell numbers through fusion events could have a large clinical significance.

Together, results from this study demonstrate that the inflammatory environment of EAE increases cellular fusion and heterokaryon formation between human and rodent cells in the cerebellum of mice after intravenous injection of human bone marrow-derived MSCs. In addition, we were able to show that fusion between MSCs and cerebellar neuronal cells occurs *in vitro*, with an increase in cellular fusion events in the presence of TNF-alpha and/or IFN-gamma. The finding that fusion between bone marrow-derived cells and cerebellar neurons is, we believe, of significant importance. Specifically, the observation that fusion events increase in response to inflammatory mediators *in vivo* and *in vitro* suggests that the process may be a useful therapeutic target for human inflammatory diseases of the CNS. The number of fusion events is small and further studies are warranted to improve understanding of the process and to test ways to increase fusion levels. As MSCs can be easily isolated from the bone marrow and rapidly expanded *in vitro*, these

© 2011 The Authors

findings suggest that bone marrow-derived stem cells may be potential therapeutic agents for cerebellar and neuroinflammatory diseases.

Acknowledgements

We would like to acknowledge the Multiple Sclerosis Society of Great Britain and Northern Ireland, the Wellcome Trust and Ataxia UK for support.

References

1 Himes BT, Neuhuber B, Coleman C, Kushner R, Swanger SA, Kopen GC, Wagner J, Shumsky JS, Fischer I. Recovery of function following grafting of human bone marrow-derived stromal cells into the injured spinal cord. *Neurorehabil Neural Repair* 2006; **20**: 278–96

2 Zhang J, Li Y, Chen J, Yang M, Katakowski M, Lu M, Chopp M. Expression of insulin-like growth factor 1 and receptor in ischemic rats treated with human marrow stromal cells. *Brain Res* 2004; **1030**: 19–27

3 Zhang J, Li Y, Lu M, Cui Y, Chen J, Noffsinger L, Elias SB, Chopp M. Bone marrow stromal cells reduce axonal loss in experimental autoimmune encephalomyelitis mice. *J Neurosci Res* 2006; **84**: 587–95

4 Gordon D, Pavlovska G, Glover CP, Uney JB, Wraith D, Scolding NJ. Human mesenchymal stem cells abrogate experimental allergic encephalomyelitis after intraperitoneal injection, and with sparse CNS infiltration. *Neurosci Lett* 2008; **448**: 71–3

5 Bang OY, Lee JS, Lee PH, Lee G. Autologous mesenchymal stem cell transplantation in stroke patients. *Ann Neurol* 2005; **57**: 874–82

6 Lee PH, Kim JW, Bang OY, Ahn YH, Joo IS, Huh K. Autologous mesenchymal stem cell therapy delays the progression of neurological deficits in patients with multiple system atrophy. *Clin Pharmacol Ther* 2008; **83**: 723–30

7 Scolding N, Marks D, Rice C. Autologous mesenchymal bone marrow stem cells: practical considerations. *J Neurol Sci* 2008; **265**: 111–5

8 Parr AM, Tator CH, Keating A. Bone marrow-derived mesenchymal stromal cells for the repair of central nervous system injury. *Bone Marrow Transplant* 2007; **40**: 609–19

9 Rice CM, Scolding NJ. Adult stem cells – reprogramming neurological repair? *Lancet* 2004; **364**: 193–9

10 Magrassi L, Grimaldi P, Ibatici A, Corselli M, Ciardelli L, Castello S, Podesta M, Frassoni F, Rossi F. Induction and survival of binucleated Purkinje neurons by selective damage and aging. *J Neurosci* 2007; **27**: 9885–92

11 Johansson CB, Youssef S, Koleckar K, Holbrook C, Doyonnas R, Corbel SY, Steinman L, Rossi FM, Blau HM. Extensive fusion of haematopoietic cells with Purkinje neurons in response to chronic inflammation. *Nat Cell Biol* 2008; **10**: 575–83

12 Nygren JM, Liuba K, Breitbach M, Stott S, Thoren L, Roell W, Geisen C, Sasse P, Kirik D, Bjorklund A, Nerlov C, Fleischmann BK, Jovinge S, Jacobsen SE. Myeloid and lymphoid contribution to non-haematopoietic lineages through irradiation-induced heterotypic cell fusion. *Nat Cell Biol* 2008; **10**: 584–92

13 Weimann JM, Charlton CA, Brazelton TR, Hackman RC, Blau HM. Contribution of transplanted bone marrow cells to Purkinje neurons in human adult brains. *Proc Natl Acad Sci U S A* 2003; **100**: 2088–93

14 Weimann JM, Johansson CB, Trejo A, Blau HM. Stable reprogrammed heterokaryons form spontaneously in Purkinje neurons after bone marrow transplant. *Nat Cell Biol* 2003; **5**: 959–66

15 Chopp M, Li Y. Treatment of neural injury with marrow stromal cells. *Lancet Neurol* 2002; **1**: 92–100

16 Mahmood A, Lu D, Lu M, Chopp M. Treatment of traumatic brain injury in adult rats with intravenous administration of human bone marrow stromal cells. *Neurosurgery* 2003; **53**: 697–702; discussion 702–3

17 Mahmood A, Lu D, Qu C, Goussev A, Chopp M. Human marrow stromal cell treatment provides long-lasting benefit after traumatic brain injury in rats. *Neurosurgery* 2005; **57**: 1026–31; discussion 1026–31

18 Blau HM. A twist of fate. *Nature* 2002; **419**: 437

19 Singec I, Snyder EY. Inflammation as a matchmaker: revisiting cell fusion. *Nat Cell Biol* 2008; **10**: 503–5

20 Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**: 143–7

21 O'Neill EJ, Day MJ, Wraith DC. IL-10 is essential for disease protection following intranasal peptide administration in the C57BL/6 model of EAE. *J Neuroimmunol* 2006; **178**: 1–8

22 Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, Hardy W, Devine S, Ucker D, Deans R, Moseley A, Hoffman R. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival *in vivo*. *Exp Hematol* 2002; **30**: 42–8

23 Beyth S, Borovsky Z, Mevorach D, Liebergall M, Gazit Z, Aslan H, Galun E, Rachmilewitz J. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood* 2005; **105**: 2214–9

24 Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringden O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol* 2003; **31**: 890–6

25 Maitra B, Szekely E, Gjini K, Laughlin MJ, Dennis J, Haynesworth SE, Koc ON. Human mesenchymal stem cells support unrelated donor hematopoietic stem cells and suppress T-cell activation. *Bone Marrow Transplant* 2004; **33**: 597–604

© 2011 The Authors

26 Bennaceur-Griscelli A, Pondarre C, Schiavon V, Vainchenker W, Coulombel L. Stromal cells retard the differentiation of CD34(+)CD38(low/neg) human primitive progenitors exposed to cytokines independent of their mitotic history. *Blood* 2001; **97**: 435–41
27 Devine SM, Bartholomew AM, Mahmud N, Nelson M, Patil S, Hardy W, Sturgeon C, Hewett T, Chung T, Stock W, Sher D, Weissman S, Ferrer K, Mosca J, Deans R, Moseley A, Hoffman R. Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. *Exp Hematol* 2001; **29**: 244–55
28 Amado LC, Saliaris AP, Schuleri KH, St John M, Xie JS, Cattaneo S, Durand DJ, Fitton T, Kuang JQ, Stewart G, Lehrke S, Baumgartner WW, Martin BJ, Heldman AW, Hare JM. Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. *Proc Natl Acad Sci U S A* 2005; **102**: 11474–9
29 Devine SM, Cobbs C, Jennings M, Bartholomew A, Hoffman R. Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. *Blood* 2003; **101**: 2999–3001
30 Kemp K, Hares K, Mallam E, Heesom KJ, Scolding N, Wilkins A. Mesenchymal stem cell-secreted superoxide dismutase promotes cerebellar neuronal survival. *J Neurochem* 2009; **114**: 1569–80
31 Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**: 315–7
32 Nordquist DT, Kozak CA, Orr HT. cDNA cloning and characterization of three genes uniquely expressed in cerebellum by Purkinje neurons. *J Neurosci* 1988; **8**: 4780–9
33 Priller J, Persons DA, Klett FF, Kempermann G, Kreutzberg GW, Dirnagl U. Neogenesis of cerebellar Purkinje neurons from gene-marked bone marrow cells *in vivo*. *J Cell Biol* 2001; **155**: 733–8
34 Bae JS, Han HS, Youn DH, Carter JE, Modo M, Schuchman EH, Jin HK. Bone marrow-derived mesenchymal stem cells promote neuronal networks with functional synaptic transmission after transplantation into mice with neurodegeneration. *Stem Cells* 2007; **25**: 1307–16
35 Bae JS, Furuya S, Shinoda Y, Endo S, Schuchman EH, Hirabayashi Y, Jin HK. Neurodegeneration augments the ability of bone marrow-derived mesenchymal stem cells to fuse with Purkinje neurons in Niemann-Pick type C mice. *Hum Gene Ther* 2005; **16**: 1006–11
36 Stoll G, Jander S, Schroeter M. Cytokines in CNS disorders: neurotoxicity versus neuroprotection. *J Neural Transm Suppl* 2000; **59**: 81–9
37 Okuda Y, Sakoda S, Bernard CC, Yanagihara T. The development of autoimmune encephalomyelitis provoked by myelin oligodendrocyte glycoprotein is associated with an upregulation of both proinflammatory and immunoregulatory cytokines in the central nervous system. *J Interferon Cytokine Res* 1998; **18**: 415–21
38 Juedes AE, Hjelmstrom P, Bergman CM, Neild AL, Ruddle NH. Kinetics and cellular origin of cytokines in the central nervous system: insight into mechanisms of myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis. *J Immunol* 2000; **164**: 419–26
39 English K, Barry FP, Field-Corbett CP, Mahon BP. IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunol Lett* 2007; **110**: 91–100
40 Lapham LW. Tetraploid DNA content of Purkinje neurons of human cerebellar cortex. *Science* 1968; **159**: 310–2
41 Mares V, Lodin Z, Sacha J. A cytochemical and autoradiographic study of nuclear DNA in mouse Purkinje cells. *Brain Res* 1973; **53**: 273–89
42 Kozorovitskiy Y, Gould E. Stem cell fusion in the brain. *Nat Cell Biol* 2003; **5**: 952–4

Received 3 March 2010
Accepted after revision 25 August 2010
Published online Article Accepted on 3 September 2010