

Short communication

An investigation of auto-reactivity after head injury

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Abstract

Murine models of CNS injury show auto-reactive T cell responses directed at myelin antigens, associated with improved neuronal survival and functional recovery. This pilot study shows, for the first time, that similar immune responses against myelin occur in human traumatic brain injury (TBI), with an expansion of lymphocytes recognising myelin basic protein observed in 40% of patients studied. “Reactive” patients did not have greater contusion volume on imaging, but were younger than the “unreactive” subgroup and tended towards a more favorable outcome. These findings are consistent with the concept of “beneficial autoimmunity”.

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1. Introduction

The normal T cell repertoire contains lymphocytes that react with self; these include encephalitogenic anti-myelin lymphocytes (Meinl et al., 1997; Pette et al., 1990a,b). In health, these myelin-reactive T cells are held in check by regulatory mechanisms. We hypothesized that after head injury the systemic release of myelin antigens might break this tolerance. This would be manifested by proliferation and expansion of the anti-myelin T cell pool. We reasoned that tolerance might be more easily broken with greater auto-antigen load, so we correlated immunological parameters with radiological measures of injury volume.

One possible outcome of anti-myelin T cell activation might be cell-mediated cerebral inflammation (Fee et al., 2003). It is possible that such inflammation might contribute to secondary injury in the acute phase; however, there is at least the prospect that it might promote repair. The concept of such “beneficial autoimmunity” has come from work on

murine models of central nervous system injury. Various studies have demonstrated that systemic autoreactive T cells, including those with a Th1 phenotype, reduce neuronal death (Ibarra et al., 2004; Kipnis et al., 2001, 2002a,c). We therefore correlated myelin autoreactivity with clinical outcome at 6 months.

2. Materials and methods

2.1. Subjects

Blood samples were obtained from 11 healthy controls (six female; mean age 38 years), and from 10 patients following severe TBI admitted to the Neurosciences Critical Care Unit at Addenbrooke's Hospital. Following assent from patient representatives, 30 ml of heparinised blood was taken within 72 h of injury and at 10 days thereafter. All patients required sedation, mechanical ventilation and the institution of a protocol-driven therapy aimed at maintaining intracranial pressure <20 mm Hg and cerebral perfusion pressure >70 mm Hg (Menon, 1999). The studies were approved by the Local Research Ethics Committee at Addenbrooke's Hospital, Cambridge, UK (LREC: 97/290).

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2.2. Injury severity

Injury severity at admission was assessed using the post-resuscitation GCS; the Glasgow Outcome Score (GOS) (Jennett and Bond, 1975) was recorded 6 months later. The physical volume of lesions was quantified on magnetic resonance images (MRI) (nine patients) and computerised tomography (CT) images (patients) obtained within 72 h of injury, using the Analyze 6.0 (AnalyzeDirect Inc., Lenexa, Kansas, USA) biomedical imaging resource. This involved using intensities to outline the core (low signal intensity) and peri-contusional (high intensity) areas on the Fluid Attenuation Inversion Recovery (FLAIR) MRI sequences (single operator JN, blinded to the immunological results) with subsequent measurement of volumes of each area (Fig. 1). Computerised tomography analysis differed only in that the core typically demonstrates high signal intensity and the peri-contusional areas low intensity.

2.3. Preparation of lymphocytes for inclusion in proliferation assays

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood by Ficoll density gradient separation (Ficoll Plaque Plus, Amersham Pharmacia Biotech) and labelled with the intracellular cell division tracking dye Carboxy fluorescein diacetate succinimidyl ester (CFSE; Vybrant CFDA SE Cell Tracer Kit V-12883, Molecular Probes) prior to inclusion in proliferation assays (Lyons et al., 2001). CFSE was added to PBMCs suspended in RPMI without serum to a final concentration of 5 μ M, incubated at room temperature for 15 min and then quenched with ice-cold RPMI containing 10% serum.

2.4. Proliferation assays

10^6 CFSE labeled PBMCs were incubated in RPMI 1640 medium with 1% penicillin, 1% streptomycin, 1% L-glutamine and 10% autologous serum in 12 well flat bottomed plates for 10 days at 37 °C. 10^6 PBMCs were also incubated under identical condition with the addition of one of the following antigens: 50 mcg/ml human myelin

basic protein (MBP), which was more than 95% pure by SDS-Page (Cat # RDI-TRK8M79, Research Diagnostics Inc.), Tuberculin purified protein derivative (PPD; Aventis Pasteur) or keyhole limpet antigen (KLH; Sigma, Cat no. H7017). Finally, under the same incubation conditions, 10^6 PBMCs were stimulated with 1 mcg/ml of soluble monoclonal anti-CD3 (Cat # 550367, Clone HIT3a, BD Pharmingen) and anti-CD28 (kind gift from Mark Frewin, Sir William Dunn School of Pathology, Oxford). At the end of the 10 day incubation period the cells were harvested, washed and fixed. Lymphocytes were incubated with anti-CD4 and anti-CD19 fluorochrome-conjugated monoclonal antibodies prior to flow cytometric analysis. The amount of CFSE fluorescence per cell, and surface labelling of CD4 and CD19 was measured using a FACScalibur flow cytometer (Becton Dickinson) and CELLQuest™ software. Precursor frequency and proliferation index were calculated using Modfit LT 3.0 (Verity Software) (Givan et al., 1999).

2.5. Real-time semi-quantification of mRNA expression

The proliferation assays described were set up in duplicate (with the exception of no CFSE labelling of lymphocytes in the duplicate set). Following the 10 day incubation period PBMCs were frozen at -70 °C in RNAlater (Ambion) according to the manufacturer's instructions. Total mRNA was subsequently extracted with RNeasy Mini Kit (QIAGEN) and converted to cDNA using Pro-STAR First Strand RT-PCR Kit (Stratagene). Primers and probes (see Table 1) were designed using the Primer Express software (PE Biosystems, Foster City, CA, USA) based on GenBank sequences (available via the National Centre for Biotechnology Information website <http://www.ncbi.nlm.nih.gov>) and purchased from Oswel DNA Service. Real-time expression of various mRNA sequences was measured with the PerkinElmer ABI Prism 7900HT Sequence Detection System using PCR Mastermix containing ROX (Eurogentec RT-QP2X-03). Primers and probes were used at a final concentration of 3 μ M and 1 μ M respectively. Gene expression was normalized against either β -Actin (IL-10 and IFN γ), or CD3 (IL-5) where expression was known to be limited to T cells (Fontenot et al., 2003; McKenzie and Sanderson, 1992).

2.6. The quantification of cytokine concentrations in supernatants

Supernatants were harvested from proliferation assays at day 10 and frozen on the day of collection to -20 °C. IFN- γ and IL-10 were measured using the Duoset human IFN- γ and Quantikine human IL-10 enzyme linked immunosorbent assay kits (R&D systems; catalog numbers DY285 and D1000B respectively). IL-4 and IL-5 was measured using the Duoset human IL-4 and IL-5 ELISA kits (R&D systems, DY205 and DY204 respectively). All

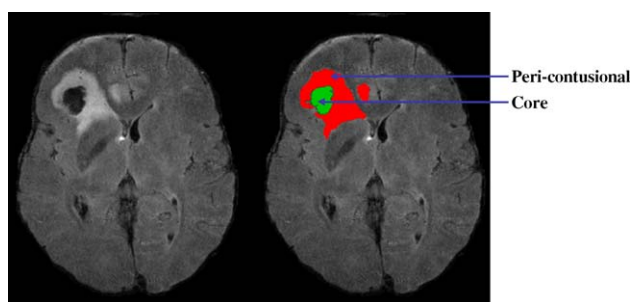


Fig. 1. Two FLAIR MRI images illustrating the injury sustained by two patients participating in the study. The left hand image demarcates the core and peri-contusional area of brain the area of which was calculated to determine the lesion volume.

Table 1
Primer and probe sequences used in this study

Gene	Forward primer	Reverse primer	Probe
β -Actin	5'-CCT GGC ACC CAG CAC AAT-3'	5'-GCC GAT CCA CAC GGA GTA CT-3'	5'-TCA AGA TCA TTG CTC CTC CTG AGC GC-3'
Interleukin-5	5'-GAC GCA GTC TTG TAC TAT GCA CTT TC-3'	5'-AGA AGC ATC CTC ATG GCT CTG A-3'	5'-TTG CCA AAG GCA AAC GCA GAA C-3'
CD3	5'-GGC CTC CGC CAT CTT AGT AA-3'	5'-GGC CCA GAA CTC TCC AGT GA-3'	5'-AAC AGT CCC ATG AAA CAA AGA TGC AGT CG-3'
Interleukin 10	5'-TGA GAA CAG CTG CAC CCA CTT-3'	5'-GCT GAA GGC ATC TCG GAG AT-3'	5'-CCA GGC AAC CTG CCT AAC ATG CTT-3'
Interferon γ	5'-CCA ACG CAA AGC AAT ACA TGA-3'	5'-TTC GCT TCC CTG TTT TAG CTG-3'	5'-CAT CCA AGT GAT GGC TGA ACT GTC GCT-3'

plates were read using a microplate reader (model 680, BioRad).

2.7. Statistical analysis

All statistical analyses were performed using SPSS 10.0 for Windows. Following assessment of normality, parametric (Student's T test) or non-parametric (Wilcoxon or Mann–Whitney) tests were performed. Bonferroni corrections for multiple comparisons were made where applicable.

3. Results

3.1. Patient demographics

Ten patients (8 male) were recruited to the study over a period of 6.5 months. The mean age was 33 years (range 19–52 years). The majority of additional medical problems involved skeletal fractures and skin lacerations. One patient developed bilateral adrenal haemorrhages. The median post-resuscitation Glasgow Coma Score (GCS) (Teasdale and Jennett, 1974) was 6 (range, 4–14).

3.2. Traumatic brain injury results in a delayed increase in the number and proliferation of lymphocytes specifically in response to myelin basic protein

PBMCs isolated from patients within 72 h of severe TBI and 10 days thereafter were incubated with self-antigen (MBP), recall antigen (PPD), neo-antigen (KLH) or nothing for 10 days. Proliferation was measured by CFSE dilution. The *precursor frequency* (defined as the proportion of lymphocytes that leave the original parent population to undergo at least two cell divisions) (Givan et al., 1999; Lyons et al., 2001) of the lymphocyte population was measured in patients and controls. There was a non-significant trend for lymphocytes taken within 72 h of TBI to have a lower precursor frequency compared to the later time point and that measured for controls (Fig. 2). There was also a trend for a greater mean precursor frequency at the later time point compared to lymphocytes harvested within 72 h (Fig. 2). The difference between the early and late time points reached significance only when PBMCs were stimulated with human MBP ($p=0.029$, Fig. 2), when the mean precursor frequency increased by 621%. As expected, polyclonal stimuli (anti-CD3 and CD28 antibodies) induced proliferation an order of magnitude

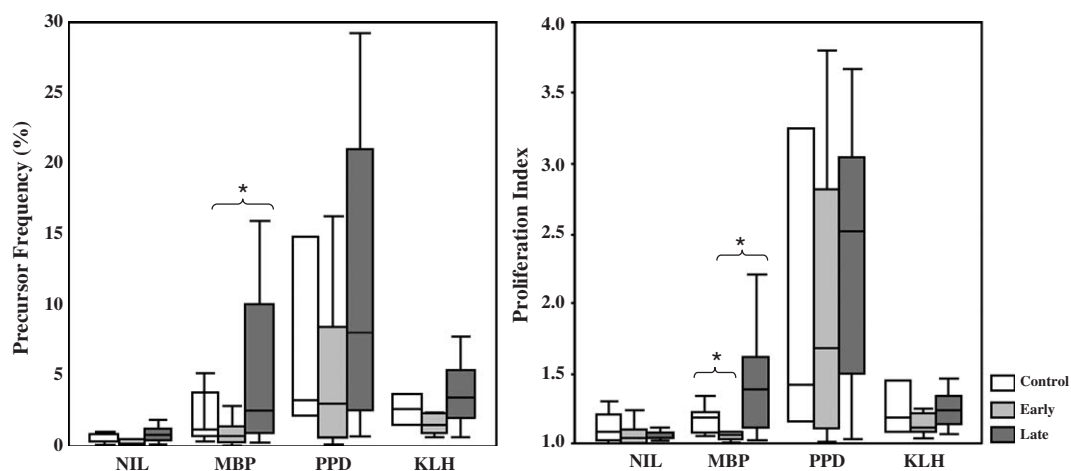


Fig. 2. The precursor frequency and proliferation index recorded after incubation of lymphocytes for 10 days with various stimuli. Controls and TBI patients are shown. 'Early' represents samples taken within 72 h of TBI; 'late' samples were taken 10 days there after. Median, quartiles and range are shown. * $p < 0.05$.

greater than the antigen-specific responses, and there was no significant difference between the head injury and control groups, and no change over time after head injury (data not shown).

A similar trend was seen when the *proliferation index* (the sum of the cells in all generations divided by the

computed number of original parental cells) was measured in the cell cultures (Fig. 2). Again only lymphocytes stimulated with MBP demonstrated an increase in mean proliferation index at the later time point compared to PBMCs taken within 72 h of TBI ($p=0.033$, Fig. 2). Interestingly, patients' lymphocytes taken at the early time

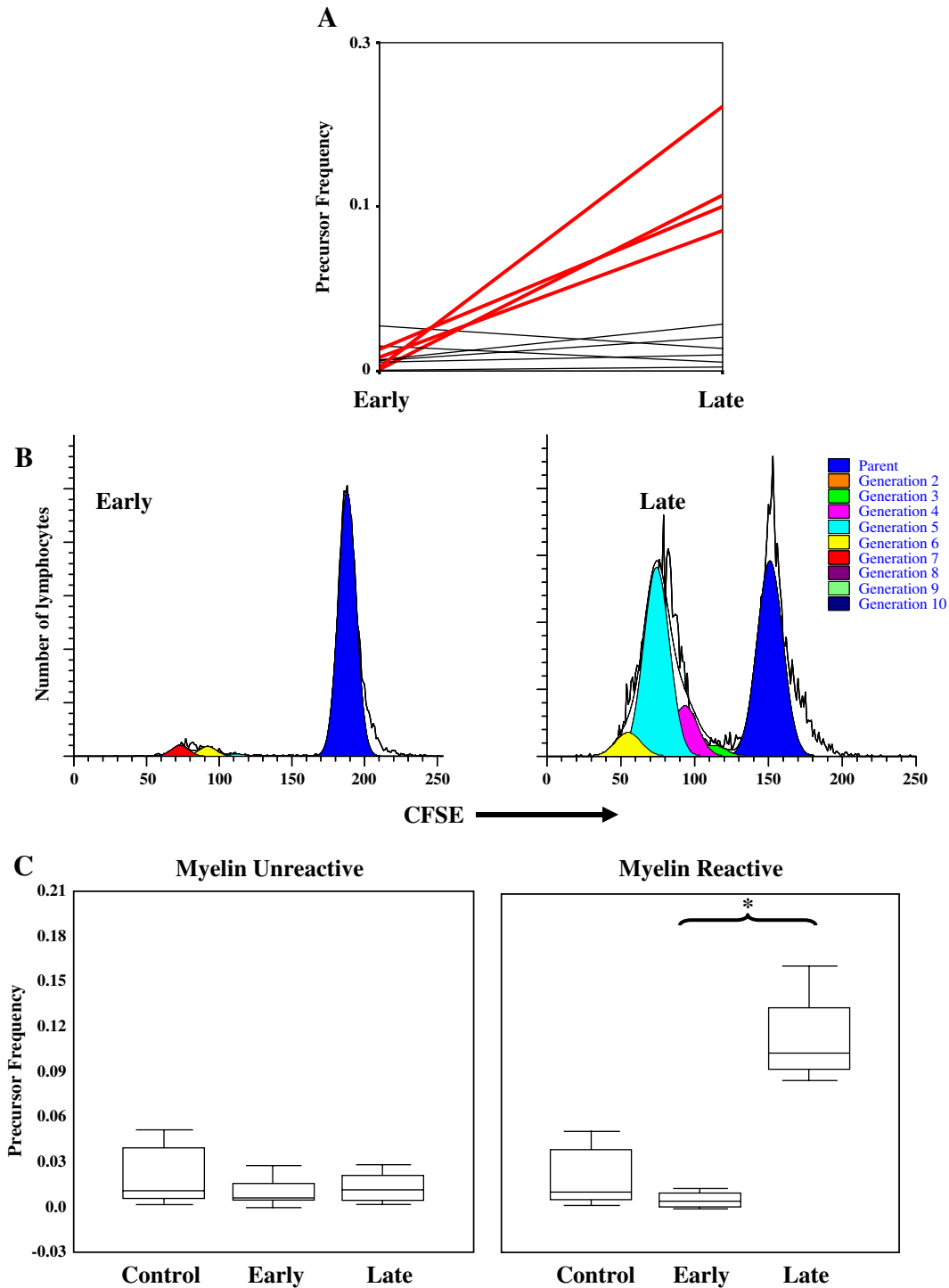


Fig. 3. Classification of patients as myelin-reactive or unreactive. (A) Precursor frequencies recorded for patients at the early and late time points. (B) The CFSE dilution histograms for a representative patient from the myelin-reactive group. (C) The precursor frequencies (median, quartiles and range) for the myelin-reactive and unreactive groups and control. $*p < 0.05$.

point proliferated significantly less than controls when stimulated with MBP, but not when other antigenic stimuli were used ($p=0.022$; Fig. 2). In particular reduced proliferation was not seen at the early time point when the recall antigen PPD was used to stimulate lymphocytes.

3.3. Only a subgroup of head injury patients expanded their myelin-reactive lymphocyte pool after head injury

It became clear when analysing the raw data (Fig. 3A) that a subset of four patients were responsible for the delayed increase in lymphocyte reactivity to myelin after TBI. We therefore classified these four patients as ‘reactive’ to MBP stimulation and compared the behaviour of their lymphocytes to the six ‘unreactive’ patients. The CFSE dilution data for a representative individual from the four reactive to myelin is shown in Fig. 3B. The level of proliferation recorded at the early time point did not predict which individuals would be myelin-reactive 10 days later (Fig. 3A). In the myelin-reactive group the precursor frequency increased by a factor of around 50 (mean 5336%, $p<0.001$; Fig. 3C). Using flow cytometric analysis of surface CD4 and CD19 expression on CFSE labelled lymphocytes, we demonstrated the proliferating lymphocytes were exclusively of a CD4+ phenotype (data not shown).

3.4. Cytokine secretion in head injury patients is suppressed by a post-transcriptional mechanism

The cytokines IFN- γ , IL-10, IL-4 and IL-5 were quantified in supernatants from PBMCs cultured for 10 days in the presence of antigens. IL-4 and IL-5 were undetectable. Concentrations of IFN- γ and IL-10 were considerably lower in the patients’ supernatants at both time points compared to controls (Fig. 4). IL-10 secretion was significantly less in patients compared to controls ($p=0.004$ at both time points) with supernatant concen-

Table 2

Treatment of head injuries

Pt	Surgical intervention	Infection	Barbiturates	Steroids	Mannitol
1	Decompressive craniectomy	Pneumonia	Yes	Yes	Yes
2	Debridement of parietal depressed skull fracture	Nil	Yes	Nil	Nil
3	Decompressive craniectomy	Sputum: <i>H. influenzae</i>	Nil	Nil	Yes
4	Nil	Nil	Nil	Nil	Yes
5	Decompressive craniectomy	Sputum, urine, blood	Nil	Nil	Yes
6	EVD	Sputum: <i>S. aureus</i>	Nil	Nil	Yes
7	Nil	Sputum: <i>S. pneumoniae</i>	Nil	Nil	Nil
8	Emergency craniotomy for ASDH	Nil	Nil	Yes	Yes
9	Nil	Sputum: MRSA	Nil	Yes	Yes
10	Nil	Nil	Nil	Nil	Yes

trations reduced by a factor of 40 (Fig. 4). This was a post-transcriptional effect as patients’ lymphocyte mRNA expression for IFN- γ , IL-5 and IL-10 was equivalent to or greater than controls (data not shown). Despite the overall suppression of cytokine secretion, PBMCs taken from patients at the later time point and stimulated with MBP tended to produce a greater quantity of both of these cytokines compared with those taken within 72 h of injury (Fig. 4). This was significant ($p=0.028$), and this effect was not seen with the other stimuli used.

3.5. Myelin reactivity was associated with youth but not injury volume or severity

We asked whether there were any clinical differences between myelin-reactive and unreactive patients. There was

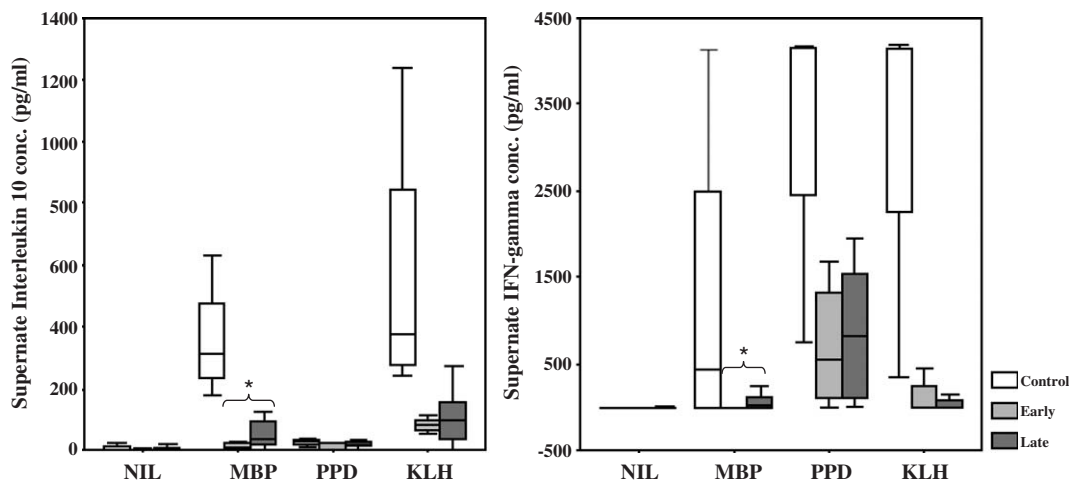


Fig. 4. Cytokine production. Median, quartiles and range of concentration of IFN- γ and IL-10 (pg/ml) in supernatants harvested after lymphocytes were left in various culture conditions for 10 days. Controls and TBI patients are shown. ‘Early’ = lymphocytes taken within 72 h of TBI; ‘late’ lymphocytes taken 10 days later.

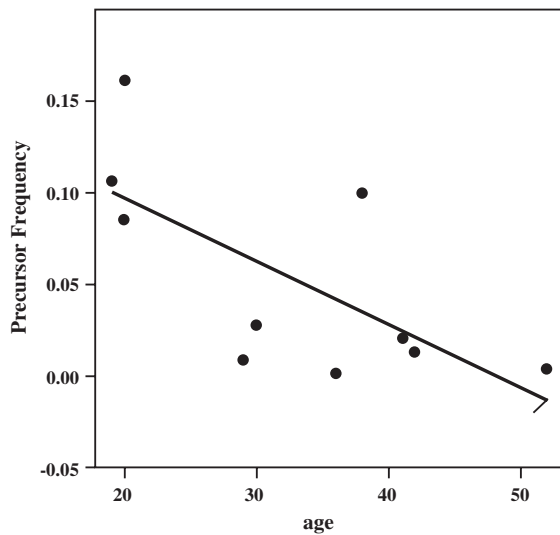


Fig. 5. Myelin reactivity correlates with age. This figure illustrates the inverse correlation that exists between the age of the patient (years; x axis) and the precursor frequency (y axis). Pearson correlation coefficient = -0.68 .

no difference in the GCS recorded at baseline (median score 5 and 7 for reactive and unreactive groups, respectively). None of the patients had previously sustained a head injury. There was a non-significant trend for greater volume of contused brain in the myelin unreactive cohort (33,551.21 mm³ (range 7368.37–61,723.92) versus 25,803 mm³ (range 8224.73–55,823.75)). Therefore the extent of neural injury produced by TBI did not influence the immunological response, nor did intercurrent infections or medical interventions, although there was a trend for the patients in the myelin-reactive group to require more aggressive therapies (surgical intervention or barbiturate therapy) for uncontrolled intracranial hypertension (Table 2).

The myelin-reactive cohort was younger than the unreactive cohort (mean age 24 versus 38 years, $p=0.037$) and there was a significant inverse correlation between age and the myelin specific precursor frequency at the later time point after TBI ($p=0.03$; $R^2=0.47$; Fig. 5). Six month follow-up data were available from 9 patients, one of the myelin-reactive groups having died at 5 months of bronchopneumonia. The median GOS for the 3 survivors in the myelin-reactive group was marginally better than the unreactive group at 4 and 3 respectively, although this was not a significant difference.

4. Discussion

We hypothesised that the exposure of lymphocytes to unusually large quantities of myelin antigens would stimulate proliferation of myelin specific lymphocytes. We demonstrated a significant increase in the number of myelin specific auto-reactive lymphocytes, and the extent to which they proliferate, after TBI. These data demonstrate for the

first time that auto-reactive T cell responses directed at myelin antigens occur in human head injury. These results replicate data from animal models (Hauben et al., 2000; Kipnis et al., 2002b,c), where such response are associated with improved outcomes, leading to the concept of “beneficial autoimmunity”.

Our sample size is too small to draw conclusions regarding the impact of such immune responses in humans, but the heterogeneity that we observe raises some intriguing possibilities. The clinical severity and radiological extent of the head injury did not correlate with myelin-reactivity. However the myelin-reactive patients were more likely (non-significantly) to have required surgical decompression or barbiturate therapy to control intracranial pressure. A trivial explanation is that neurosurgery increases brain antigen exposure to the immune system and hence myelin reactivity. An alternative possibility is that myelin reactivity drives a cerebral inflammatory response and exacerbates the cerebral oedema associated with head injury (Fee et al., 2003). However, the three survivors from the myelin-reactive group tended to have better outcome scores than the unreactive group, perhaps consistent with animal data indicating a beneficial role for myelin reactivity in promoting repair in the post-acute phase (Kipnis et al., 2002c). Further studies are required to confirm these early findings, and to explore their potential pathophysiological and therapeutic implications.

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