Complex Autoantibody Responses Occur following Moderate to Severe Traumatic Brain Injury

Edward J. Needham, Oda Stoevesandt, Eric P. Thelin, Henrik Zetterberg, Elisa R. Zanier, Faiez Al Nimer, Nicholas J. Ashton, Joanne G. Outtrim, Virginia F. J. Newcombe, Hani S. Mousa, Joel Simrén, Kaj Blennow, Zhihui Yang, Peter J. Hutchinson, Fredrik Piehl, Adel E. Helmy, Mike J. Taussig, Kevin K. W. Wang, Joanne L. Jones, David K. Menon and Alasdair J. Coles

KEY POINTS

TBI triggers autoantibody production..

Alterations in autoantibody repertoire persist for years postinjury.

Abstract

Most of the variation in outcome following severe traumatic brain injury (TBI) remains unexplained by currently recognized prognostic factors. Neuroinflammation may account for some of this difference. We hypothesized that TBI generated variable autoantibody responses between individuals that would contribute to outcome. We developed a custom protein microarray to detect autoantibodies to both CNS and systemic Ags in serum from the acute-phase (the first 7 d), late (6–12 mo), and long-term (6–13 y) intervals after TBI in human patients. We identified two distinct patterns of immune response to TBI. The first was a broad response to the majority of Ags tested, predominantly IgM mediated in the acute phase, then IgG dominant at late and long-term time points. The second was responses to specific Ags, most frequently myelin-associated glycopeptide (MAG), which persisted for several months post-TBI but then subsequently resolved. Exploratory analyses suggested that patients with a greater acute IgM response experienced worse outcomes than predicted from current known risk factors, suggesting a direct or indirect role in worsening outcome. Furthermore, late persistence of anti-MAG IgM autoantibodies correlated with raised serum neurofilament light concentrations at these time points, suggesting an association with ongoing neurodegeneration over the first year postinjury. Our results show that autoantibody production occurs in some individuals following TBI, can persist for many years, and is associated with worse patient outcome. The complexity of responses means that conventional approaches based on measuring responses to single antigenic targets may be misleading.

Introduction

Traumatic brain injury (TBI) is the leading cause of death and disability in young adults in the developed world (1). Despite significant advances in knowledge there are few reliable early prognostic markers, and there is marked heterogeneity in outcome between individuals with seemingly similar initial primary injuries. Indeed, the best established prognostic models in TBI (such as the CRASH outcome prediction calculator and International Mission for Prognosis and Analysis of Clinical Trials [IMPACT] models) explain less than 40% of outcome variance (2). Secondary injury, the process in which (among others) metabolic and inflammatory consequences of TBI cause additional neurologic injury, is likely to contribute significantly to this heterogeneity and is potentially

therapeutically modifiable (3). In addition, TBI is now increasingly believed to trigger a chronic neurodegenerative process in a subset of patients (4–13).

The mechanisms underlying early secondary injury and late neurodegeneration are not well understood, but neuroinflammation has been implicated in both processes and represents a potential therapeutic target. Most work to date has focused on innate immunity and microglial activation, which may persist decades after injury. The intensity of the late microglial response, in particular, appears to correlate with late functional outcome and white matter damage (14–16). However to date, therapeutic modulation of these systems has not had any clinical impact (17, 18).

In addition to local innate immune activation, TBI disrupts both brain tissue and the blood–brain barrier (BBB), releasing brain Ags into the systemic circulation and into the cervical lymph nodes via glymphatic and meningeal lymphatic systems (19, 20), generating both humoral and cellular adaptive autoimmune responses, which may be detrimental (21–23). The phenomenon of destructive autoimmunity triggered by CNS injury is well established, with notable examples including sympathetic ophthalmia and NMDAr encephalitis following herpes simplex encephalitis (24, 25). As far back as the 1960s, studies of small patient cohorts have described autoantibodies to individual brain Ags following TBI (21, 23, 26–29). Given the plethora of different brain Ags released after TBI, it is unlikely that measuring a single autoantibody captures the true extent of autoimmune response. Indeed, although the cognate Ags have been poorly characterized, past studies show that autoantibodies to multiple CNS targets are likely to be produced following TBI (21).

The function and clinical relevance of these autoantibodies are uncertain. They may represent an important mechanism for clearing cellular debris (30); however, there are also suggestions from experimental spinal cord injury (SCI) that they may be pathogenic. Injection of sera from mice with SCI into the hippocampi of uninjured mice induced glial activation with prominent neuron loss, whereas sera from B cell knockout mice that had also undergone SCI had no such effect. Furthermore, B cell competent mice with SCI developed ectopic meningeal lymphoid follicles, resembling those seen in patients with multiple sclerosis, providing mechanistic insights into how traumatic adaptive immune activation could cause ongoing CNS injury, even after the BBB had been reconstituted after injury (31).

We hypothesized that the release of brain Ags into the systemic circulation and cervical lymph nodes following injury, in the context of a heightened danger-associated molecular pattern milieu, generates autoantibodies against a variety of brain proteins, which may be pathogenic. To screen for such autoantibodies, we developed a CNS human protein microarray, with brain and nonbrain Ags, and analyzed samples from the acute phase and at two late time points after moderate to severe TBI. We hypothesized that autoantibodies present in the acute phase would associate with worse clinical outcome at 6–12 mo post-TBI, and their persistence in the longer term would associate with biomarkers of ongoing neurodegeneration.

Materials and Methods

Study populations

Written consent for TBI patients was obtained from legal representatives prior to enrolment in the acute phase, and further written consent for ongoing use of data and study participation were obtained from the patients at follow up. The studies described were approved either by the Cambridgeshire local research ethics committee (REC 97/290 and 13/EE/0119) or regional ethical board in Stockholm (no. 2005/1526/31/2).

Healthy controls were recruited through the University of Cambridge (REC 97/290) and Cambridge Biomedical Research Centre (REC 11/33/0007), and all provided written consent. A further bank of 28 "positive control" samples from patients with autoimmune thyroid disease, type 1 diabetes, multiple sclerosis, or autoimmune encephalitis was provided by Sanja Ugrinovic (Department of Immunology, Addenbrookes Hospital, U.K.) and Patrick Waters (Nuffield Department of Clinical Neurosciences, University of Oxford, U.K.), which were used in the early development of the protein microarray contributed normative data.

Procedures

Sample collection and storage

Serum samples were collected at up to four different time points: 1) "acute," within 72 h of injury (before an adaptive immune response should have occurred); 2) "subacute," 7 d postinjury; 3) "late," 6–12 mo postinjury; and 4) "long-term," 6–13 y postinjury. A number of individuals donated samples at multiple time points; a schematic diagram of the samples used is shown in Supplemental Fig. 1A. The samples were aliquoted, labeled with pseudoanonymized identifiers, and frozen immediately at −70°C. Samples from the validation cohort were shipped on dry ice with temperature monitoring to the University of Cambridge.

Demographic, clinical, and outcome information

Demographic information and IMPACT score variables [age, postresuscitation Glasgow Coma Scale motor score, pupil reactivity, occurrence of hypoxia or hypotension, Marshall computerized tomography (CT) classification (32), presence of subarachnoid or epidural hemorrhage on CT, blood glucose and hemoglobin concentration (33)] were recorded by the research team at the time of admission. Injuries were characterized using the Injury Severity Scale (34). Glasgow Outcome Score (GOS) Extended (35) was recorded for the discovery cohort at 6–9 mo post-TBI, and GOS (36) was recorded for the validation cohort at 9–13 mo post-TBI, as per the respective original protocols for both of these studies.

Autoantibody screening

Autoantibody screening was undertaken using a custom protein microarray based on the HuProt (version 2.0) platform (37). The microarray was devised in collaboration with Cambridge Protein Arrays (Cambridge, U.K.) and CDI Laboratories (Puerto Rico) to detect autoantibodies to a broad selection of CNS, BBB, and systemic Ags. The microarrays consisted of a glass microscope slide with a thin SuperEpoxy coating, printed with quadruplicate spots of recombinant yeast-expressed whole

proteins, each fused with glutathione-S-transferase (GST). The array included 79 targets: 52 brain related, 5 BBB related, and 22 nonbrain related (full Ag list detailed in Supplemental Table I). Each slide accommodated up to 12 individual serum samples. Samples from the same patient at different time points (acute, subacute, and late) were assayed on the same slide. The long-term (6–13 y postinjury) cohort was run in a separate batch alongside healthy controls; the 28 age- and sex-matched healthy controls used as their comparators were recruited contemporaneously.

In brief, the slides were blocked in 2% BSA/0.1% PBS-Tween overnight at 4°C, washed, and then incubated with 200 µl of 1:1000 diluted serum at room temperature for 2 h. The slides were washed again, incubated at room temperature for 2 h with fluorophore-conjugated goat anti-human IgM-µ chain-Alexa488 (catalog no. A21215; Invitrogen, Carlsbad, CA) and goat anti-human IgG-Fc-DyLight550 (catalog no. SA5-10135; Invitrogen) secondary Abs, washed, and then scanned using a Tecan LS400 scanner and GenePix Pro v4 software, with the output being median fluorescence value of the quadruplicate spots for each protein. Supplemental Fig. 2A–C demonstrate the reproducibility of microarray results. Ag-specificity of the Ab binding is shown in Supplemental Fig. 2D.

Anti-GFAP autoantibody ELISA

To assess the impact of the yeast expression system used for the protein microarray, verification of the anti-GFAP autoantibody signal seen on the protein microarray was performed using a custom ELISA with Escherichia coli–expressed protein. The 96-well ELISA plates were coated with 100 µl of 100 ng/ml recombinant E. coli–expressed GFAP (catalog no. DXAG-001; Dx-SyS, Mountain View, CA) and incubated overnight at 4°C. The plates were blocked with 200 µl of blocking buffer (catalog no. 37542; Thermo Fisher Scientific) for 2 h at 37°C with constant rocking, washed three times with 200 µl of TBST, and then incubated with 100 µl of dilute serum (1:100 in blocking buffer) for 2 h at room temperature. After a further three washes, the plates were incubated with 100 µl of dilute (1:10000) HRP-conjugated anti-human IgM (catalog no. 109-036-129; Jackson Immunoresearch) or IgG (catalog no. 109-036-098; Jackson Immunoresearch) Ab for 1 h and washed three times, and 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) solution was added (catalog no. 34028; Thermo Fisher Scientific). After 5 min, 100 µl of TMB stop solution (catalog no. N600; Thermo Fisher Scientific) was added, and the plates were read with a Tecan infinite M200 Pro ELISA reader set to 450 nm (correction wavelength 540 nm), taking a blank well as zero.

Ig fraction isolation

To determine whether the microarray results represented binding of Ab or some other serum component (38), IgM and IgG were isolated from samples using κ and Λ magnetic beads (PureProteome). Serum was diluted with PBS (25 µl to a volume of 100 µl) and incubated with 300 µl of bead slurry (prepared according to the manufacturer's instruction) for 60 min at room temperature with continuous end-over-end rotation. The beads were separated using a magnetic rack, and the immunodepleted serum was removed. The Ig fraction was eluted from the beads using three washes with 150 µl of 0.1 M glycine HCl, and the resultant eluent was neutralized with 45 µl of 1 M Tris HCl. Estimation of IgG recovery in the eluent was estimated by NanoDrop to be ∼50%, and thus the eluent was diluted to the equivalent of 1 in 500 before being run as standard on the protein microarray, so as to ensure that the Ig concentration closely approximated that of 1 in 1000 sera. The

immunodepleted serum was run as standard on the protein microarray having compensated for the additional dilution.

Total Ig quantification

Total IgG and IgM were measured using the standard clinical assay at Addenbrookes Hospital, U.K.

Neurofilament light and glial fibrillary acidic protein quantification

Acute GFAP concentrations in the discovery cohort were measured by Randox Laboratories Ltd (Crumlin, U.K.), using a sandwich chemiluminescent immunoassay (Evidence Investigator Cerebral Custom Array IV). Serum samples were transported on dry ice.

For quantification of NfL and GFAP concentrations in the validation, late, and long-term cohorts, serum samples were shipped on dry ice to the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, where the analyses were performed using commercially available kits (NF-Light Advantage and GFAP discovery kit, respectively) on the single molecule array (Simoa) platform, according to instructions from the manufacturer (Quanterix, Billerica, MA)

Statistical analysis

Descriptive data are presented using median and interquartile range (for continuous variables) or number and percentage (for categorical variables). Between group differences were calculated using Mann–Whitney U tests for unpaired continuous variables and Wilcoxon matched-pairs signed rank tests for paired continuous variables; complete statistics for all comparisons are tabulated in Supplemental Table II. The association between two continuous variables was assessed using linear regression. Categorical data were compared using $\chi 2$ analysis. Variance of groups was compared using an F test. Temporal profiles of paired samples were assessed using Friedman with post hoc Dunn tests. All p values stated are unadjusted and two tailed. The Benjamini–Hochberg procedure, with a false discovery rate set to 10%, was used to account for multiple comparisons when appropriate, and p values that remained <0.05% after correction are highlighted.

Outcome prediction

The risk of poor outcome was calculated for the acute cohorts using the IMPACT prognostic calculator Core + CT + Lab model (http://www.tbi-impact.org/?p=impact/calc). When individual covariate data were not available (n = 2% of values; all relating to laboratory results), a value was imputed by using the median value of all patients in that cohort. To assess whether autoantibody responses were associated with outcome, we used a sliding dichotomy approach (39) to group patients according to whether their outcome was “worse than expected” or “better than/as expected” compared with the predictions made by the IMPACT Calculator (Supplemental Fig. 1B, 1C).

Protein microarray

The degree of Ab binding was quantified by the median fluorescence of the four quadruplicate spots for each Ag. Fluorescence values for each Ag were then normalized by dividing by the median value of all Ags for that patient. Both raw (nonnormalized) and normalized values were then converted into Z-scores based on respective normal distributions generated from data for each Ag from 269 samples (all samples from the discovery and validation cohorts, healthy controls and "positive" controls); the top and bottom 2% of values for each Ag were excluded to remove artifactual outliers or strong positive results so that the interpretation of Z-scores approximates those of a Gaussian distribution. This broad selection of subjects included both "well" and critically ill participants to ensure it would include variation attributable to nonspecific binding resulting from acute-phase reactants (38). Although this approach may have reduced sensitivity to detection of Ab responses, we accepted this as a price for the increased specificity that resulted. A representative distribution of Z-scores within all samples assayed is displayed in Supplemental Fig. 1D. For the initial screen, a positive autoantibody result was defined by a threshold of $Z \geq 3$. An increase of $Z \geq 1$ between paired samples, providing the second sample showed a $Z \geq 3$, was used to define the development of a new autoantibody.

Analyses were performed using GraphPad Prism (version 8.1.0; GraphPad Software) and SPSS (version 25; IBM SPSS).

Data availability

Data from this study are available to academic collaborators on application to the corresponding author.

Results

Study populations and measurements

Banked samples of serum collected at up to two time points during the first week of admission (acute: 0–3 d post-TBI and subacute: 7–10 d post-TBI) from two retrospective cohorts of patients with moderate to severe TBI, recruited from two separate centers, were studied sequentially as discovery and validation cohorts. The discovery cohort (n = 25) was recruited from patients admitted to the Neurosciences Critical Care Unit, Addenbrookes Hospital, U.K., between January 2012 and August 2013, and the validation cohort (n = 66) was recruited from patients admitted to the Neurointensive Care Unit, Karolinska University Hospital, Sweden, between January 2007 and October 2012. The demographic details of both acute cohorts are summarized in Table I. The validation cohort was older ($p = 0.001$) and more likely to have a mass lesion ($p = 0.001$) and traumatic subarachnoid hemorrhage ($p = 0.001$) but had lower overall trauma severity (based on the Injury Severity Scale; $p = 0.0003$). They had a poorer predicted outcome based on the IMPACT variables ($p < 0.0001$), but GOS at follow up was not different ($p = 0.125$). The validation cohort was followed up at a median of 12 mo postinjury compared with 7 mo for the discovery group.

A subset of the discovery cohort (n = 20) provided serum samples 6–12 mo postinjury, forming a third late cohort. A fourth long-term cohort (n = 34, 13 of whom had contributed to the discovery

cohort, and 6 of whom had samples at all four time points) provided new serum samples 6–13 y post-TBI (see Supplemental Fig. 1A for details of sample time points for individual patients).

Healthy controls (n = 45) were recruited through the University of Cambridge and Cambridge Biomedical Research Centre (demographic comparisons with relevant cohorts shown in Table I).

Acute upregulation of IgM and IgG responses

IgM Abs to most Ags were considerably upregulated between the acute (day 0–3 post-TBI) and subacute (day 7) time points (n = 20, $p < 0.0001$), with a smaller increase in IgG ($p = 0.035$) (Fig. 1A, 1B; Supplemental Table II). These findings from the discovery cohort were replicated in the validation cohort ($p < 0.001$ and $p = 0.003$, for IgM and IgG; Supplemental Table II). At day 7, there was a greater variation in IgM response between subjects compared with acute samples ($F = 0.409$, $p = 0.004$; (Fig. 1C). There was no similar effect in the IgG response ($F = 1.16$, $p = 0.75$; (Fig. 1D). Total serum IgM was increased in half of patients (Supplemental Fig. 1E) and correlated with the median IgM Z-score derived from the protein microarray ($R2 = 0.62$, $p < 0.001$) (Fig. 1E). Following immunodepletion, the median IgM signal reduced to the level of the control subunits on the microarray (i.e., subunits processed in the absence of serum) (Fig. 1F). In comparison, total serum IgG was low, with 13 out of 25 (52%) of patients' values below normal reference levels (Supplemental Fig. 1F), and there was no relationship with mean IgG Z-score on the protein microarray (Supplemental Fig. 1G).

New autoantibodies develop in the acute phase of TBI

To identify Ags driving high autoantibody responses, the microarray fluorescence data were normalized (see Materials and Methods), and new autoantibody responses were defined as an increase of Z-score ≥ 1 between paired samples, providing the second sample showed a $Z \geq 3$. Five of twenty patients in the discovery cohort (25%) developed new IgM autoantibodies, and 13 out of 20 (65%) patients developed new IgG autoantibodies between the acute and subacute time points (Fig. 2A). We confirmed these responses to be specific to Ig binding to its cognate Ag (Supplemental Fig. 2D). To exclude that these findings were related to the yeast expression of proteins, we performed ELISA using E. coli–expressed GFAP to confirm the presence of anti-GFAP autoantibodies, to which both IgM and IgG were seen on the protein microarray. The ELISA IgM signal was largely dominated by the broad upregulation of IgM (as measured by the median IgM Z-score across all Ags on the protein microarray); the strong anti-GFAP IgG signal seen on the protein microarray was replicated in the IgG ELISA (Fig. 2B).

The presence of particularly strong responses to individual Ags was replicated in the validation cohort, in which new IgM and IgG autoantibodies were seen in 42 out of 66 (64%) and 37 out of 66 (56%) patients, respectively.

At the subacute time point, Ags with high IgG responses were almost 10 times more likely to also have an IgM response compared with Ags with a low IgG response (5 out of 16 [31%] versus 25 out of

568 [4.4%], odds ratio [OR] 9.87, p = 0.0001). In contrast, at the acute time point, there was no such relationship (1 out of 11 [9%] versus 20 out of 538 [2.6%], OR = 2.52, p = 0.37).

After combining the discovery and validation cohorts, autoantibodies were seen to 51 different Ags (44 IgM, 31 IgG), of which, 24 out of 51 (47%) Ags induced both IgM and IgG responses. The most frequently seen overall targets were MAG, COL5a2, CLDN5, GFAP, and SELE (Fig. 2E). Although CNS targets comprised most of the 15 most commonly seen autoantibodies (9 out of 15 IgM, 7 out of 15 IgG), autoantibodies to BBB Ags (TJP1, CLDN5, and SELE) were also common (3 out of 15 for both IgM and IgG). There were also autoantibodies to systemic Ags (3 out of 15 IgM, 5 out of 15 IgG); we commonly observed responses against COL5a2, a ubiquitous collagen, autoantibodies against which are recognized in respiratory disease and implicated in rejection of lung transplants (40, 41). In the discovery group (where trauma CT series reports were available), the subacute IgG response to COL5a2 was higher in patients with lung contusions than those without (p = 0.04, Supplemental Table II).

Unexpectedly, autoantibodies appeared to be more likely to develop against extracellular or plasma membrane–expressed proteins than those restricted to the intracellular compartment, both for CNS (IgM OR 2.04, p = 0.02; IgG OR 1.3, p = 0.32) and non-CNS (IgM OR 3.95, p = 0.01; IgG OR 3.87, p = 0.006), once the relative number of Ags in each compartment was accounted for.

Having identified these Ags through a stringent threshold of $Z \geq 3$ autoantibody response, we compared Z-scores for the five most frequently seen autoantibodies for every patient. Four out five IgM responses (against COL5a2, GFAP, SELE, and MAG) and 1/5 IgG responses (against MAG) were increased in the subacute group compared with the acute samples (Fig. 2C, 2D).

To assess the relationship between acute protein release and subsequent autoantibody response, the acute serum GFAP concentration was compared with subacute GFAP autoantibody responses. A significant relationship was seen in the discovery cohort (Spearman rho 0.58; p = 0.008). However, in the validation cohort, with an alternative GFAP assay, this finding was not replicated (Spearman rho 0.19; p = 0.12).

Subacute IgM autoantibody responses may be associated with worse late clinical outcome

The discovery and validation cohorts were pooled to investigate the association of autoantibody responses with clinical parameters. Younger age was associated with a higher IgM response (R2 = 0.19, p < 0.0001), but none of the following parameters correlated with either IgM or IgG responses: sex, Glasgow Coma Scale motor score, Injury Severity score, and IMPACT score.

In the 25 patients from the discovery cohort, those with an outcome worse than predicted had a higher median IgM Z-score at day 7 than those whose outcome was as good as, or better than, expected (p = 0.01) (Fig. 1G; Supplemental Table II). The median IgM Z-score at day 7 could

differentiate these two groups (area under the curve 0.776, p = 0.019; (Fig. 1H). There was no difference between groups in the number of new IgM or IgG autoantibodies (p = 0.70).

In the validation cohort, however, there was no relationship between any autoantibody profile and outcome (median IgM Z p = 0.15; number of normalized IgM Abs Z ≥ 3 p = 0.67; number of normalized IgG Abs Z ≥ 3 p = 0.33) (Supplemental Table II). However, the older age of this cohort meant that the IgM response was less marked; the clinical outcome was less granular, as only GOS had been collected (Supplemental Fig. 1C); and the GOS was recorded at a median of 12 mo rather than 7.

IgM and IgG autoreactivity persist for several years postinjury

A subset of 20 patients from the discovery cohort provided samples at a late time point (6–12 mo post-TBI, Supplementary Table IB), which had significantly higher median nonnormalized Z-scores for both IgM and IgG than both controls (p < 0.0001 for both; Supplemental Table II) and their acute samples (p < 0.0002 for IgM and p < 0.0001 for IgG, respectively; (Fig. 3A, 3B; Supplemental Table II). The IgM response peaked at the subacute time point before falling toward (but not back to) baseline, whereas IgG reactivity was maximal at the late time point (p < 0.0001 for all comparisons except acute versus subacute IgG, in which p = 0.058).

Temporal profiles of autoantibody responses. (A) Broad IgM upregulation peaking at the subacute time point (day 7 post-TBI), and waning over the ensuing months to years, but never returning to baseline. (B) Broad IgG upregulation peaking at the late time point (6–12 mo post-TBI) and remaining at this level long-term (6–13 y post-TBI). (C) The temporal profiles of dominant anti-MAG IgG autoantibodies from three patients with serum taken at four time points at late time points (6–9 mo) showing a return toward baseline long-term (7–13 y). (D and E) Screening for the five most frequently seen autoantibodies in the acute phase reveals late persistent IgM to MAG and IgG to MAG and SELE. Healthy control (HC) group 1, acute, subacute, and late samples were assayed contemporaneously in one experimental batch; HC group 2 and long-term samples were assayed contemporaneously as a separate experimental batch. Statistical tests for paired TBI samples: Wilcoxon matched-pairs signed rank test; for controls versus TBI: Mann–Whitney U test.

A further long-term cohort who had sustained a moderate to severe TBI between 6 and 13 y previously (n = 34, of whom 13 had contributed to the discovery cohort) were screened for autoantibodies and showed persistent upregulation of IgM and (particularly) IgG compared with healthy controls (p = 0.016 and p < 0.0001 for IgM and IgG, respectively; (Fig. 3A, 3B; Supplemental Table II).

The five most frequently detected autoantibodies seen during the acute phase were assessed in the late (6–12 mo post-TBI) samples after normalization. There were persistent IgG autoantibodies against MAG and SELE but also IgM autoantibodies to MAG (Fig. 3D, 3E). However, these autoantibodies were not seen in the long-term samples (p > 0.05 for all comparisons with controls at this time point; Supplemental Table II), suggesting a waning of response over years (Fig. 3C).

To assess whether late IgG autoantibodies were the result of subacute class-switching, the data from the 11 patients with samples taken at acute, subacute, and late time points were analyzed for temporal relationships. Those Ags in which a late IgG $Z \geq 3$ was seen were more likely to have a corresponding subacute IgM of $Z \geq 2$ (this lower threshold again used to capture more-subtle responses) than those Ags in which late IgG $Z < 3$ (7 out of 27 [26%] versus 58 out of 919 [6%], OR 5.2, $p < 0.0001$). There was no such relationship seen between late IgG and acute IgM (1 out of 27 [4%] versus 38 out of 919 [4%], OR = 0.89, $p = 0.91$), suggesting that the late IgG are indeed related to subacute IgM production.

Markers of neurodegeneration are seen in a subset of patients years after TBI and differ depending on autoantibody profile

Both NfL and GFAP concentrations were significantly higher at a group level in the late TBI (6–12 mo postinjury) cohort than healthy controls (NfL $p < 0.0001$; GFAP $p = 0.05$; (Fig. 4A; Supplemental Table II), and there was an association between the concentrations of the two proteins ($R2 = 0.3$, $p = 0.002$; (Fig. 4B). Although the effective half-life of NfL in the serum has not been fully delineated (but believed to be in the region of a few weeks), the effective half-life of GFAP is between 24 and 48 h (42), and thus the presence of raised GFAP levels, although less marked, is highly suggestive of an active injurious process, rather than slow clearing of protein released at the time of injury. Although at the long-term (6–13 y) time point there was no significant group difference in serum GFAP or NfL concentration between TBI patients and healthy controls (GFAP $p = 0.11$, NfL $p = 0.4$; (Fig. 4C, Supplemental Table II), a larger proportion of TBI patients had neural injury biomarkers above the control normal range (defined as values within 2 SD of the control population mean) (GFAP 7 [20%] versus 1 [0.25%], $p = 0.01$; NfL 7 [20%] versus 2 [0.5%], $p = 0.046$), suggesting that at least a subset of patients experience ongoing neurodegeneration, in keeping with previous studies (4, 7–9, 11, 13).

As NfL appeared to be the more sensitive of the two biomarkers to discriminate from healthy controls, the late autoantibody profiles were regressed against serum NfL concentrations. Although no association survived adjustment for multiple comparisons, two hypotheses were generated by this analysis: 1) high anti-MAG IgM reactivity associates with high NfL, and 2) median IgG Z-scores associate with low NfL (Fig. 4D). High anti-MAG IgM weakly correlated with high NfL ($R2 = 0.15$, $p = 0.029$) in the late-term cohort, but this relationship was largely driven by a single individual.

Discussion

In this study, we identified two discrete autoantibody responses following TBI.

The first is an upregulation of (initially) predominantly IgM autoantibodies against many Ags, commencing within the first week of TBI but persisting for several years. This picture is reminiscent of an alteration in the “natural autoantibody” repertoire, which are low-affinity polyreactive species produced by B1 cells, and thought to have, among other functions, a role in the clearance of cellular debris (43). That such a response might occur acutely in response to massive cell injury is easy to appreciate and in keeping with murine experiments, which suggest that circulating autoantibodies following TBI bind to dying neurons (30). However, the ongoing alteration of this system years after the injury is surprising, and its implications as yet unknown. This polyantigenic IgM upregulation was so marked in some patients that it could be detected by measuring total serum IgM concentration. In

young patients, a large IgM response appeared to be an independent predictor of worse outcome; although the intention of this response may be to clear the debris of cell death, it is conceivable that in severe trauma, the subsequent immune-complex load could be detrimental. If the association with worse outcome is borne out in larger studies, measuring total serum IgM concentration could represent a useful prognostic biomarker and perhaps a marker for stratifying patients for immunomodulatory studies. This response diminished with increasing age, in keeping with the known effect of ageing on both B-1 (polyreactive IgM) and B-2 (Ag-specific) responses (44, 45), and so may be of less importance in older patients.

The second autoantibody response comprises a disproportionate rise in Ab concentration against a small number of neural Ags (of which MAG was the most common) but also to BBB Ags (TJP1, CLDN5) and systemic Ags (such as COL5a2). In keeping with our results, Western blot experiments (21) have also identified GFAP as a frequent autoantibody target after TBI. The temporal profile of these responses to specific Ags largely recapitulated a typical primary adaptive immune response, with early IgM and later IgG production. The primacy of MAG as an autoantigen at late time points is particularly interesting given that white matter tract degeneration is a key phenomenon driving late posttraumatic neurodegeneration (11, 16, 46), and the association with serum NfL concentrations in our cohort suggests that these autoantibodies may be playing a role in this process, although we cannot judge whether they are contributing to ongoing damage or part of the repair effort.

Previous studies addressing the production of autoantibodies following TBI have largely looked for responses to a single Ag, such as S100b (23), myelin basic protein (26), and β tubulin (28), and have found a response to the target in question. Our data, in keeping with data from Western blot experiments (21) suggest that autoantibodies are produced to a multitude of specific targets following TBI, and so measuring responses to a single Ag gives an inadequate representation of the repertoire. Furthermore, it appears that there is a marked, separate response of Abs that bind to the majority of proteins on the microarray, reminiscent of polyreactive natural Abs; this response dominated the IgM signal on ELISA and therefore would compromise the validity of results generated by single-Ag assays.

There are two main limitations of this study. First, the protein array platform uses proteins expressed by yeast and therefore may not present the target proteins in an authentic conformation. It may therefore have a lower sensitivity for certain proteins than other Ab detection techniques and conversely may detect Abs that would not bind to authentic human proteins. Despite this caveat, as demonstrated 1) by use of purified Ig fractionated from sera and 2) through preincubation with cognate Ags, results obtained from the protein microarray were shown to be specific and reproducible; furthermore, results were replicated by ELISA using protein from a different expression system, and one of the major Ab targets detected was GFAP, replicating Western blot experiment results using human brain lysate (21). The corresponding rise in total serum IgM concentration additionally validates the broad upregulation of IgM detected on the protein microarray and highlights the strength of the protein microarray technology to investigate the complexity of autoantibody responses, even if it does not have the accuracy of assays designed to detect autoantibodies to a specific single Ag using authentic human protein.

The second limitation is the baseline differences between the clinical cohorts, especially the fewer young patients in the validation cohort, which reduced the power to investigate the association between the upregulated IgM response and clinical outcome.

In conclusion, we have used protein microarray technology to screen for novel autoantibody production following moderate to severe TBI. This approach has elucidated two distinct patterns of response: 1) a diffuse upregulation of mainly IgM responses, which peaks at day 7, and which persist for years postinjury, and 2) a much greater autoantibody response to a few specific Ags that follow a vaccination-like temporal profile and persist for months but return to baseline years postinjury. Our data would suggest that the polyantigenic IgM response in the acute phase may be detrimental to clinical outcome and persistent anti-MAG IgM autoantibodies associate with surrogate markers of late neurodegeneration.

Disclosures

H.Z. has served at scientific advisory boards for Denali, Roche Diagnostics, Wave, Samumed, and CogRx; has given lectures in symposia sponsored by Fujirebio, Alzecure, and Biogen; and is a cofounder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures–based platform company at the University of Gothenburg. A.J.C. received honoraria and travel expenses from Genzyme (a Sanofi company) until September 2017. V.F.J.N. reports personal fees from Neurodiem, outside the submitted work. D.K.M. reports grants, personal fees, and nonfinancial support from GlaxoSmithKline Ltd.; grants, personal fees, and other from NeuroTrauma Sciences; grants and personal fees from Integra Life Sciences; personal fees from Pfizer Ltd.; grants and personal fees from Lantmannen AB; from Calico Ltd.; personal fees from Pressura Neuro Ltd.; and others from Cortirio Ltd., outside the submitted work. M.T. is the founder and CEO of Cambridge Protein Arrays Ltd., a company that performs Ab specificity profiling. The other authors have no financial conflicts of interest.

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Abbreviations used in this article

BBB

blood–brain barrier

CT

computerized tomography

GOS

Glasgow Outcome Score

IMPACT

International Mission for Prognosis and Analysis of Clinical Trials

OR

odds ratio

SCI

spinal cord injury

TBI

traumatic brain injury

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