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Human brain trauma severity is associated with lectin complement pathway activation

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Abstract

We explored the involvement of the lectin pathway of complement in post-traumatic brain injury (TBI) pathophysiology in humans. Brain samples were obtained from 28 patients who had undergone therapeutic contusion removal, within 12 h (early) or from >12 h until five days (late) from injury, and from five non-TBI patients. Imaging analysis indicated that lectin pathway initiator molecules (MBL, ficolin-1, ficolin-2 and ficolin-3), the key enzymes MASP-2 and MASP-3, and the downstream complement components (C3 fragments and TCC) were present inside and outside brain vessels in all contusions. Only ficolin-1 was found in the parenchyma of non-TBI tissues. Immunoassays in brain homogenates showed that MBL, ficolin-2 and ficolin-3 increased in TBI compared to non-TBI (2.0, 2.2 and 6.0-times) samples. MASP-2 increased with subarachnoid hemorrhage and abnormal pupil reactivity, two indicators of structural and functional damage. C3 fragments and TCC increased, respectively, by 3.5 - and 4.0-fold in TBI compared to non-TBI tissue and significantly correlated with MBL, ficolin-2, ficolin-3, MASP-2 and MASP-3 levels in the homogenates. In conclusion, we show for the first time the direct presence of lectin pathway components in human cerebral contusions and their association with injury severity, suggesting a central role for the lectin pathway in the post-traumatic pathophysiology of human TBI.

Keywords

Traumatic brain injury, complement system, lectin complement pathway, neuroinflammation, MBL-associated serine proteases

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Introduction

Traumatic brain injury (TBI) is a common cause of death and disability among young and old people worldwide.[1,2] In patients who survive the primary biomechanical impact, the secondary injury – caused by the activation of several molecular and cellular cascades – is the main contributor of brain damage.[3–5] Secondary injury is closely associated with activation of the inflammatory response. The complement system, an important branch of the innate immunity response, is

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a major coordinator of post-traumatic neuroinflammation and secondary neuropathology after TBI.[6–10] This system includes a heterogeneous mixture of fluid-phase and cell-associated proteins that upon activation: (1) increase blood–brain barrier (BBB) leakage via C3a and C5a; (2) favor leukocyte infiltration into the injured brain and subsequent free radical production; (3) induce neuronal and glial apoptosis via C3a and C5a binding to their receptors; and (4) promote neuronal lysis via the terminal complement complex (TCC).[10–12] Thus, therapeutic strategies aimed at blocking complement activation could potentially reduce neuroinflammation and neurodegeneration in TBI patients.[13–16]

Depending on the danger signals, the complement system can be activated by three pathways: the classical, the alternative and/or the lectin pathway, each composed of specific initiators and effector enzymes.[12] Substantial involvement of the classical pathway in post-traumatic pathology has been excluded on the basis of studies on mice genetically deficient for C1q, the initiator molecule of the classical pathway, which showed neurological deficits and lesion size similar to wild-type mice when subjected to TBI.[17] There is evidence that the lectin pathway plays a pathogenic role in acute brain injury, including stroke and TBI, in line with its ability to recognize and bind altered self-structures.[14,18–23] In humans, the lectin pathway uses mannose-binding lectin (MBL), ficolins (ficolin-1, -2 and -3) as well as collectin-10 and -11 as initiator molecules.[24] They act as soluble pattern recognition receptors that circulate in complexes with MBL-associated serine proteases (MASP-1, MASP-2 and MASP-3).[12,25] On specific binding to patterns of carbohydrates (MBL and collectins) or acetylated residues (ficolins) exposed on the surface of damaged cells (damaged associate molecular patterns, DAMPs), in addition to that of microorganisms (pathogen-associated molecular patterns, PAMPs), the lectin pathway initiators trigger the conversion of zymogen MASPs into their active state, promoting complement activation.[25–28] This leads to downstream formation of the C3 split products C3a and C3b and activation of the terminal pathway with release of C5a and the formation of terminal C5b-9 complement complex (TCC). TCC exists as the lytic membrane attack complex and a soluble non-lytic form sC5b-9.

Our group previously demonstrated that mice genetically deficient for MBL or treated with Polyman9 (a newly synthesized polymannosylated compound able *in vitro* to inhibit MBL binding to mannan residues) present attenuated sensorimotor deficits up to four weeks post-TBI.[13,14] Polyman9-treated TBI mice also showed enhanced neurogenesis and preservation of astrocytic endfeet at the contusion site,[13] suggesting lectin pathway initiators have a direct detrimental role on brain cells. In clinical TBI, high MBL[21] and low ficolin-3[29] circulating levels appear to be associated with the injury severity and clinical outcome. Evidence obtained in experimental TBI suggests that lectin pathway activators may have a role in clinical TBI being present not only in the serum compartment – activating the circulating complement cascade – but also in the brain where they may also have a direct local effect. In this study, we provide evidence of the involvement of lectin pathway components in human TBI pathology, assessing their presence and localization in brain contusions, their ability to activate the pathway and the association with the severity of injury.

Materials and methods

Study design

Brain samples were obtained from 28 patients with TBI admitted to the neuroscience ICU of the Fondazione IRCCS Ca' Granda–Ospedale Maggiore Policlinico, who underwent therapeutic neurosurgical intervention involving the removal of contused tissue (Supplementary Table 1). Biopsies obtained within 12 h from injury were considered "early" (21), while those obtained from >12 h until five days as "late" (7). Immediately after removal, the tissue that appeared necrotic and frankly hemorrhagic (contusion core) on a gross eye examination was separated from the contused surrounding tissue (pericore). These latter portions were collected and immediately stored at −80°C until use.[30] Brain samples from two non-TBI patients operated for brain tumors (glioma) and three autopsies from individuals who died of extracranial causes (collected at the Azienda Ospedaliero-Universitaria Parma) were used as non-TBI controls (Supplementary Table 2). The study on human tissue samples was approved by the local research ethics committee of the Fondazione IRCCS Ca' Granda–Ospedale Maggiore Policlinico (Session 28 January 2005, with final deliberation on 4 February 2005) and the Azienda Ospedaliero-Universitaria Parma (Session 10 December 2015, with final deliberation on 11 February 2016). The study was conducted according to Helsinki declaration and to the national ethical guidelines for the good clinical practice (D.M. 15 luglio 1997) that are in compliance with the European Union guidelines (CPMP/ICH/135/1995). Informed consent for using human samples for research purposes was obtained from the next of kin. Outlier values were handled as reported in the 'Statistical analysis' section.

Immunofluorescence and confocal analysis

Immunofluorescence was done on 20-μm coronal sections. Sections were thawed by 5 min washing with 0.05 M triphosphate-buffered saline (TBS) at room

temperature (RT) and then post-fixed by 15 min incubation with 4% PAF. After thorough washings with PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4 at RT), sections were incubated with blocking solution (10% normal goat serum, 0.3% Triton) for 1 h at RT and then with primary antibodies in the same solution overnight at 4°C. Primary antibodies used were: mouse anti-human iC3b/C3b (clone 3E7, 1: 100, a kind gift provided by Drs. Ronald Taylor and Margaret Lindorfer, University of Virginia School of Medicine),[31] mouse anti-human TCC (clone aE11, 1:100[32]), mouse anti-human MBL (1:50, Abcam, UK), mouse anti-human Ficolin-1 (1:50 FCN-166[33]), mouse anti-human Ficolin-2 (1:50, FCN-219[33]), mouse anti-human Ficolin-3 (1:50, Hycult Biotechnologies), mouse anti-human MASP-2 (1:50, Hycult Biotechnologies, The Netherlands) and mouse anti-human MASP-3 (clone 7D8, 1:50[33]). Sections were then incubated with a biotinylated secondary anti-mouse antibody (1:200, Vector Laboratories, UK) for 1 h at RT, followed by fluorescent signal coupling with a streptavidin TSA amplification kit (fluorescein or cyanine 5, Perkin Elmer, MA, USA). Alexa647- or Alexa488-conjugated Isolectin B4 from Griffonia Simplicifolia (1:100, 2 h at RT, Invitrogen, MA, USA) was used to label vessels. Sections were then incubated with True-Black quencher (1:20 in 70% Ethanol, Biotium, USA) to quench non-specific fluorescent signals. Appropriate negative controls were run without the primary antibodies. None of the immunofluorescence reactions gave any unspecific fluorescence signal in the negative controls (Supplementary Figure S1). Immunofluorescence was acquired using a scanning sequential mode to avoid bleed-through effects with an IX81 microscope equipped with a confocal scan unit FV500 with three laser lines: Ar-Kr (488 nm), He-Ne red (646 nm), and He-Ne green (532 nm, Olympus, Tokyo, Japan) and an UV diode. Three-dimensional images were acquired over a 10 µm z-axis with a 0.23 µm step size and processed using Imaris software (Bitplane, Zurich, Switzerland) and Photoshop cs2 (Adobe Systems Europe Ltd). Semi-quantitative investigation of complement protein localization in cerebral tissues was done on three fields of view sized 184 × 138 × 10 µm per patient, two patients per group. The analysis was done independently by three investigators blinded to the experimental group. The data reported in Table 1 are the median of the three evaluations.

Table 1. Semi-quantitative investigation of complement protein localization in cerebral tissues.

	Vascular		Parenchymal	
	Non-TBI	TBI	Non-TBI	TBI
MBL	−	++	−	−
Ficolin-1	++	+++	++	++
Ficolin-2	+	+++	−	++
Ficolin-3	+	+++	−	++
MASP-2	+	+++	−	++
MASP-3	+	+++	−	−
iC3b/C3b	+	+++	−	++
TCC	++	+	−	+++

Note: The distribution of complement proteins in relation to IB4 labeled blood vessels was assessed on the basis of confocal microscopy images (Figures 1, 2, 4 to 7). Scores were assigned blinded to the patient's group, as follows: − = no positivity, + = low positivity, ++ = intermediate positivity, +++ = high positivity. Three fields of view sized 184 × 138 × 10 µm per patient, two patients per group. TBI: traumatic brain injury.

Tissue homogenization

Seventy milligrams of each frozen cerebral sample were homogenized in 1% Triton X-100 lysis buffer supplemented with protease (1 × complete protease inhibitor cocktail, CPIC, Roche, USA) and phosphatase (1 µM 4-nitrophenyl phosphate 4-NPP, Roche, USA) inhibitors.[34] Homogenate was then centrifuged for 15 min at 10,000 r/min at 4°C and stored at −80°C until use.

ELISA for lectin pathway mediators

Ninety-six-well Nunc Maxisorb microtiter plates were coated with 1 µg/well mannan (for hMBL and MASP-2), or with 2.5, 12.5, 0.5 µg/well acetylated BSA (for ficolin-1, ficolin-2 or MASP-2 and ficolin-3, respectively) diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Residual protein binding sites were saturated by incubating the plates with 1% BSA (bovine albumin serum)–TBS blocking buffer (1% (w/v) BSA in 10 mM Tris-HCl, 140 mM NaCl, 1.5 mM NaN_3, pH 7.4), for 1 h RT.[35] The ELISA plates were then washed with washing buffer (TBS with 0.05% Tween 20 and 5 mM $CaCl_2$). Homogenates from brain specimens were thawed on ice and solutions of 50% (for MBL or ficolins) or 25% (for MASP-2) final homogenate concentration prepared in barbital-buffered saline (BBS; 4 mM barbital, 145 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) on ice. Wells receiving only BBS buffer were used as negative controls. Homogenate solutions were incubated on mannans or on acetylated BSA as described previously.[35] The plates were then washed and incubated for 1 h 30 min at RT with mouse polyclonal anti-human MBL (HM2061, Hycult Biotechnologies, The Netherlands), anti-human ficolin-1 FCN-166 [33]), anti-human ficolin-2 (FCN-219[33]), anti-human ficolin-3 (HM2089, Hycult Biotechnologies, The Netherlands),

anti-human MASP-2 (HM2190, Hycult Biotechnologies, The Netherlands) antibodies diluted 1:100 in washing buffer. After washing, the plates were incubated for 1 h 30 min at RT with an HRP labeled goat anti-mouse IgG antibody (Santa Cruz, TX, USA) diluted 1:1000 in washing buffer. After washing, the assay was developed by adding 100 µL substrate solution TMB (TMB Substrate Kit; code 34021; Thermo Scientific, MA, USA; 1:1 con H_2O_2 solution). The reaction was stopped by adding 100 µL H_2SO_4 2 M and absorption at OD450 nm was measured using the InfiniteM200 spectrofluorimeter managed by Magellan software (Tecan, CH). MASP-3 levels in homogenates (1:8 dilution) were measured by ELISA according to the method described in Skjoedt et al.[36] C3 fragments (iC3b/C3b) were measured by ELISA. Briefly, plates were coated with polyclonal anti-C3 antibody diluted 1:9600 in TBS. After 2 h incubation with blocking buffer (see above), brain homogenates diluted 1:8 in BBS were incubated for 1 h at 37°C. The plates were then washed and incubated for 1 h 30 min at RT with mouse anti-human iC3b/C3b (1: 500, 3E7, a kind gift provided by Drs. Ronald Taylor and Margaret Lindorfer, University of Virginia School of Medicine).[31] After washing, the plates were incubated with an HRP labeled goat anti-mouse IgG antibody and developed by TMB as described above. TCC was measured by ELISA as described previously.[37] Briefly, brain homogenates were diluted 1:2 in PBS with 10 mM EDTA and 0.05% Tween 20 and incubated on ELISA plates coated with a monoclonal antibody (aE11) specific for a neo-epitope exposed when C9 is incorporated into the TCC. For detection, a monoclonal biotinylated anti-C6 antibody (clone 9C4) and subsequent enzyme-linked streptavidin were used.

Statistical analysis

All quantifications were done by investigators blinded to patients' clinical information. Column analysis after patient stratification was done by Mann–Whitney test after examination of the data distribution with the Shapiro–Wilk normality test. Correlations were then done by computing the Spearman r. As the non-TBI data sets for MASP-3, iC3b/C3b and TCC showed a possible outlier, Dixon's Q-test for small data sets was applied using the formula for r_{10} with a critical value of $\alpha = 0.05$,[38,39] and the outlier was excluded from statistical analysis (Supplementary Table 3). Statistical analysis was done using standard software packages GraphPad Prism (GraphPad Software Inc., USA, version 6.0). All tests were two-sided and p values lower than 0.05 were considered statistically significant. Details of the statistical analysis for each experiment are reported in figure legends.

Results

Patients

Twenty-eight patients, 17 males and 11 females, were included in this study (Supplementary Table 1). The median age was 58. All these patients had severe TBI as documented by the post-stabilization motor Glasgow Coma Scale (GCS) median score of 4 and needed surgical intervention for contusion removal, either early (within 12 h, 21 patients) or late (from >12 h until 5 days, 7 patients) after admission. The causes of TBI were motor vehicle accidents (7 patients), falls (18) or assaults (3). Six-month outcomes were assessed with the Glasgow Outcome Scale (GOS) in 23 of the 28 patients. Eighteen patients had an unfavorable outcome (GOS: 1–3) and five either good recovery or moderate disability (GOS: 4–5). Brain samples from non-TBI patients are listed in Supplementary Table 2.

The lectin pathway initiators MBL, ficolin-1, ficolin-2 and ficolin-3 are present in TBI contusions

MBL was present in early and late removed cerebral contusions (Figure 1(a) and (b)). As demonstrated by the xy plane views with z projections in the confocal images and 3D renderings, MBL was mostly localized inside and outside cerebral vessels, with no difference between early and late removed contusions. Immunofluorescence for ficolin-1, ficolin-2 and ficolin-3 showed their presence in TBI specimens. All ficolins were located near cerebral vessels and also in the parenchyma with no differences in distribution between early (Figure 1(c), (e) and (g)) and late (Figure 1(d), (f) and (h)) samples. No MBL staining was found in non-TBI specimens (Figure 2(a)). Ficolin-1 was present in non-TBI cerebral tissues (Figure 2(b)), where it was located near cerebral vessels and in brain parenchyma, as further supported by 3D renderings. Ficolin-2 (Figure 2(c)) and ficolin-3 (Figure 2(d)) levels were low in non-TBI cerebral tissues, mostly located near cerebral vessels.

The lectin pathway initiators MBL, ficolin-2 and ficolin-3 are increased in TBI contusions

Plates coated with mannans or acBSA (the best ligands for MBL and ficolins, respectively) were incubated with human cerebral homogenates to quantify MBL or ficolins. MBL, ficolin-2, and -3 (Figure 3(a), (c) and (d)) were 2.0, 2.2 and 6.0 times higher, respectively, in homogenates from TBI than non-TBI specimens ($p < 0.05$ and $p < 0.01$). No difference was found for ficolin-1 (Figure 3(b)).

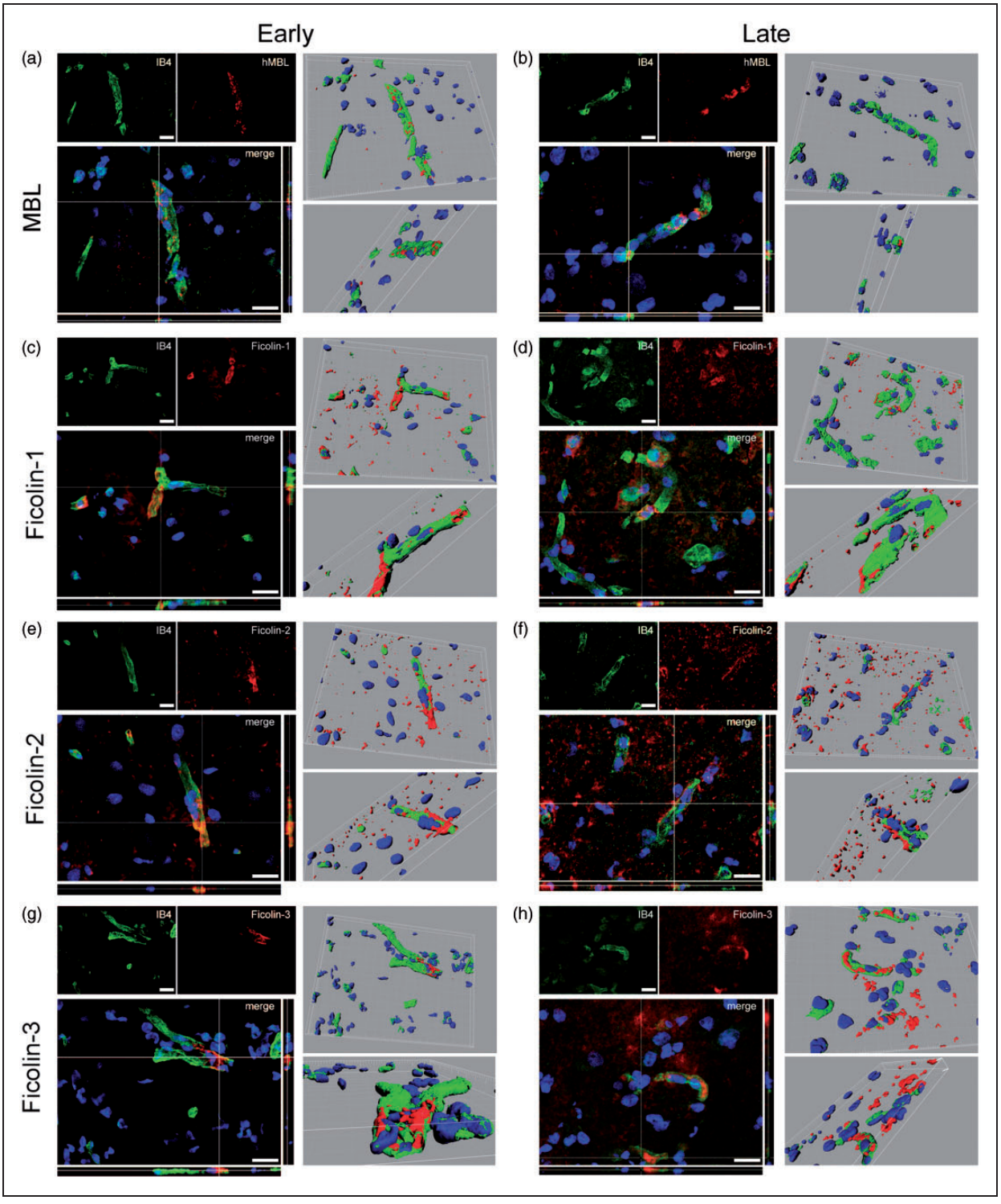

Figure 1. Lectin pathway initiators are present in human cerebral contusions after TBI. Representative images of human MBL (*red*, a, b), ficolin-1 (*red*, c, d), ficolin-2 (*red*, e, f), ficolin-3 (*red*, g, h), vessels (IB4, *green*) and nuclei (Hoechst, *blue*) in the contusional tissues removed early (a, c, e, g) or late (b, d, f, h) after TBI. Single xy plane views with z projections (merge image) and 3D renderings are presented. MBL appears mainly present inside cerebral vessels both in early (a) and late (b) removed contusions. Ficolins are present inside and near cerebral vessels and in cerebral parenchyma early (c, e, g) and in late (d, f, h) samples. Images are representative of at least two specimens per group. Scale bars 20 μm, thicks in 3D renderings 10 μm.

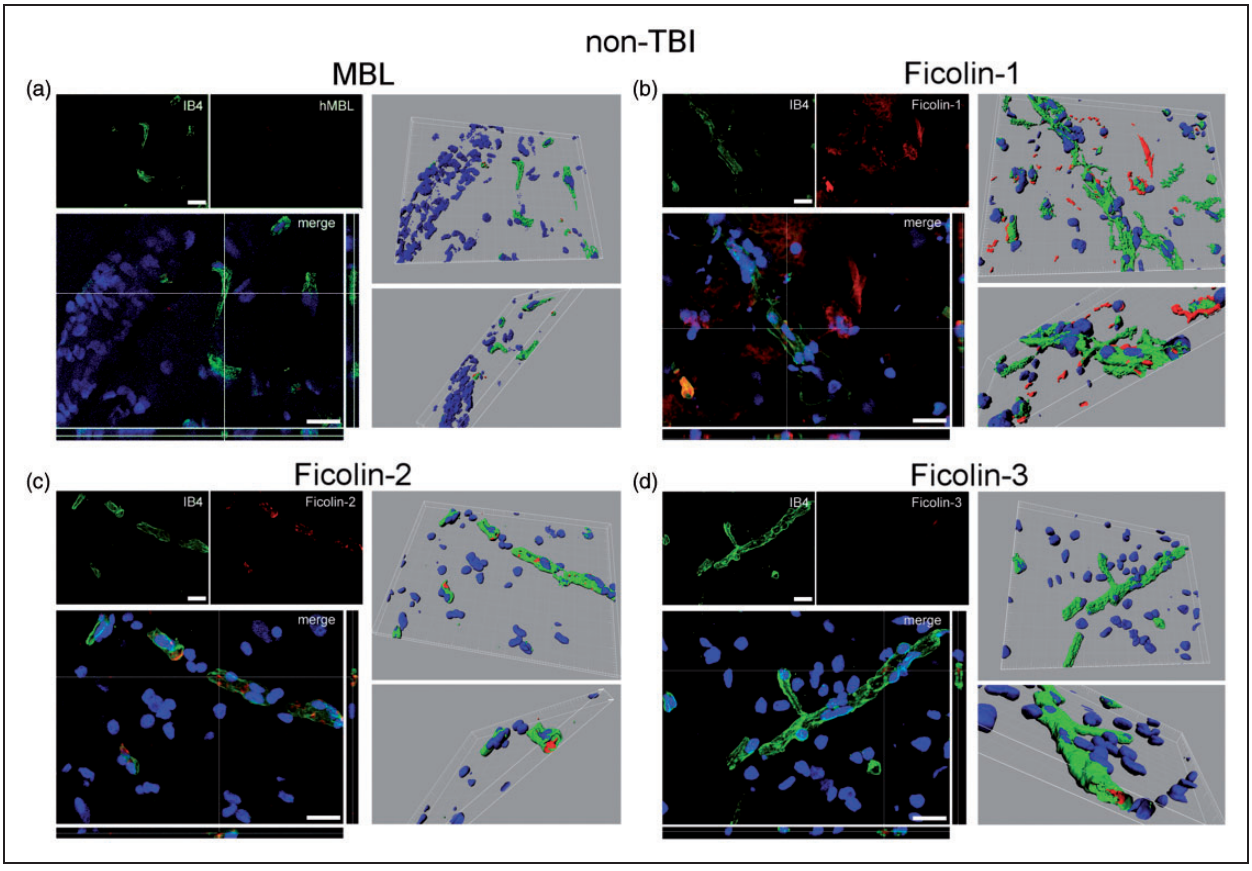

Figure 2. Lectin pathway initiators present different patterns in non-TBI human cerebral tissues. Representative images of human MBL (*red*, a), ficolin-1 (*red*, b), ficolin-2 (*red*, c), ficolin-3 (*red*, d), vessels (IB4, *green*) and nuclei (Hoechst, *blue*) in non-TBI cerebral tissues. Single xy plane views with z projections (merge image) and 3D renderings are presented. MBL is hardly detectable in non-TBI cerebral tissues (a). Ficolin-1 is present nearby cerebral vessels and in parenchyma (b). Ficolin-2 (c) and -3 (d) are scarcely present inside or nearby cerebral vessels. Images are representative of at least two specimens. Scale bars 20 μm, thicks in 3D renderings 10 μm.

MASP-2 is present in TBI and non-TBI cerebral tissues and its levels are associated with more severe clinical conditions

We focused on MASP-2, the key enzyme driving lectin pathway activation in cerebral injury.[40] MASP-2 staining was observed both in TBI (Figure 4(a) and (b)) and in non-TBI (Figure 4(c)) specimens. In early contusions, MASP-2 was located near cerebral vessels and in brain parenchyma (Figure 4(a)), while in late ones, staining was mostly in cerebral parenchyma (Figure 4(b)). In non-TBI tissues, MASP-2 staining was mostly located near cerebral vessels (Figure 4(c)). We then quantified MASP-2 deposition on plates coated with mannans (Figure 4(d) to (f)) or acBSA (Figure 4(g) to (i)) to establish the extent of MASP-2 deposition by MBL or ficolins. Homogenates from TBI specimens showed a tendency toward an increase in MBL-dependent MASP-2 deposition compared to non-TBI ones (Figure 4(d)). In addition, patients with traumatic subarachnoid hemorrhage (tSAH, Figure 4(e)) or altered pupils' reactivity (one or none reactive, Figure 4(f)) had significantly increased MBL-dependent MASP-2 levels compared to patients without tSAH or with normal pupil reactivity (1.8-fold or 1.7-fold, respectively). MASP-2 deposition driven by ficolins was significantly higher (6.7-fold) in homogenates from TBI than in non-TBI specimens (Figure 4(g)), with no association with TBI severity (Figure 4(h) and (i)).

Six-month clinical outcome (by GOS) was available for 23 of the 28 patients (Supplementary Table 1). When lectin pathway protein brain levels were stratified in relation to outcome categories,[41] no clear pattern was found (Supplementary Figure 2).

MASP-3 is present in TBI and non-TBI cerebral tissues and its levels are increased in TBI contusions

MASP-3 staining was observed in every specimen but was stronger in TBI (Figure 5(a) and (b)) than non-TBI specimens (Figure 5(c), Table 1). In every case, MASP-3 was located mainly in cerebral vessels. Homogenates

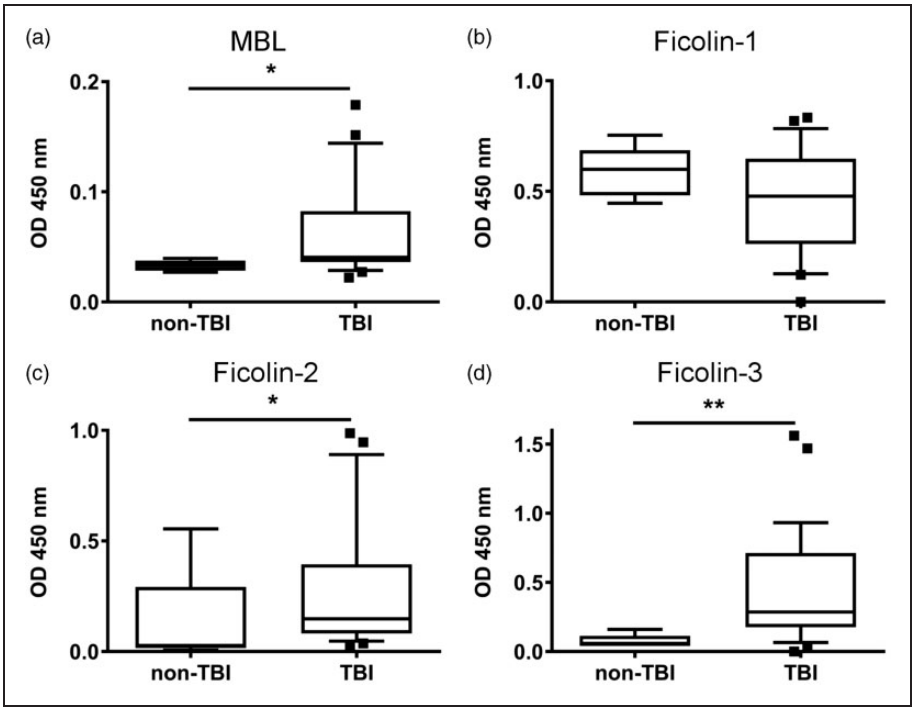

Figure 3. MBL, ficolin-2 and -3, but not ficolin-1, in homogenates of human cerebral contusions increase after TBI. MBL (a), ficolin-2 (c) and ficolin-3 (d) levels are significantly higher in TBI ($n = 28$) than non-TBI ($n = 5$) patients. Data are reported as box plots and 10th and 90th percentiles. Mann–Whitney test: $^{*}p < 0.05$; $^{**}p < 0.01$.

from TBI specimens showed a 2.1-fold increase in MASP-3 (Figure 5(d)), with no association with TBI severity (Figure 5(e) and (f)).

Downstream products of complement activation (C3 fragments, TCC) are deposited in TBI contusions

Downstream along the complement cascade, we focused on C3 active fragments (iC3b/C3b) resulting from C3 cleavage and on TCC, the final component of the complement cascade. C3 active fragments were detected in every contusion (Figure 6(a) and (b)). As supported by 3D renderings, they were localized both inside and outside cerebral vessels and in the brain parenchyma, with no difference between early and late removed contusions, indicating acute and persistent complement activation after the injury. TCC was present in cerebral contusions as a measure of full complement activation. TCC staining was found both in early (Figure 6(c)) and in late (Figure 6(d)) contusions. It was present around cell bodies in the cerebral parenchyma, indicating the formation of the lytic membrane attack complex version of TCC. In non-TBI cerebral tissues, C3 active fragments (Figure 6(e)) and TCC (Figure 6(f)) were in close association with the cerebral vessels, with no parenchymal presence. iC3b/C3b and TCC levels in brain homogenates, measured by ELISA, were, respectively, 3.5 and 4.0-times higher in TBI compared to non-TBI controls (Figure 6(g) and (h)) suggesting that TBI favors full activation of the complement system.

The immunostaining and confocal microscopy results are summarized in Table 1 which compares the different distributions of the complement components in TBI and non-TBI patients. Thus, ficolin-2 and -3, MASP-2, C3 fragments and TCC showed parenchymal presence only in TBI patients. Only MBL localized selectively on vessels and was present only in TBI specimens. MASP-3 was localized on vessels in every specimen.

Correlations between brain levels of lectin proteins and C3 fragments (iC3b/C3b) or TCC

The significant positive correlations of MBL, ficolin-2, ficolin-3, MASP-2 and MASP-3 with iC3b/C3b and/or TCC (Supplementary Table 4) indicate that the lectin and possibly the alternative pathway contribute to brain full complement activation in TBI contusions.

Discussion

This study demonstrates that in human brain contusions: (1) the complement system is fully activated down to the level of the TCC formation and depends on the lectin and possibly on amplification via the

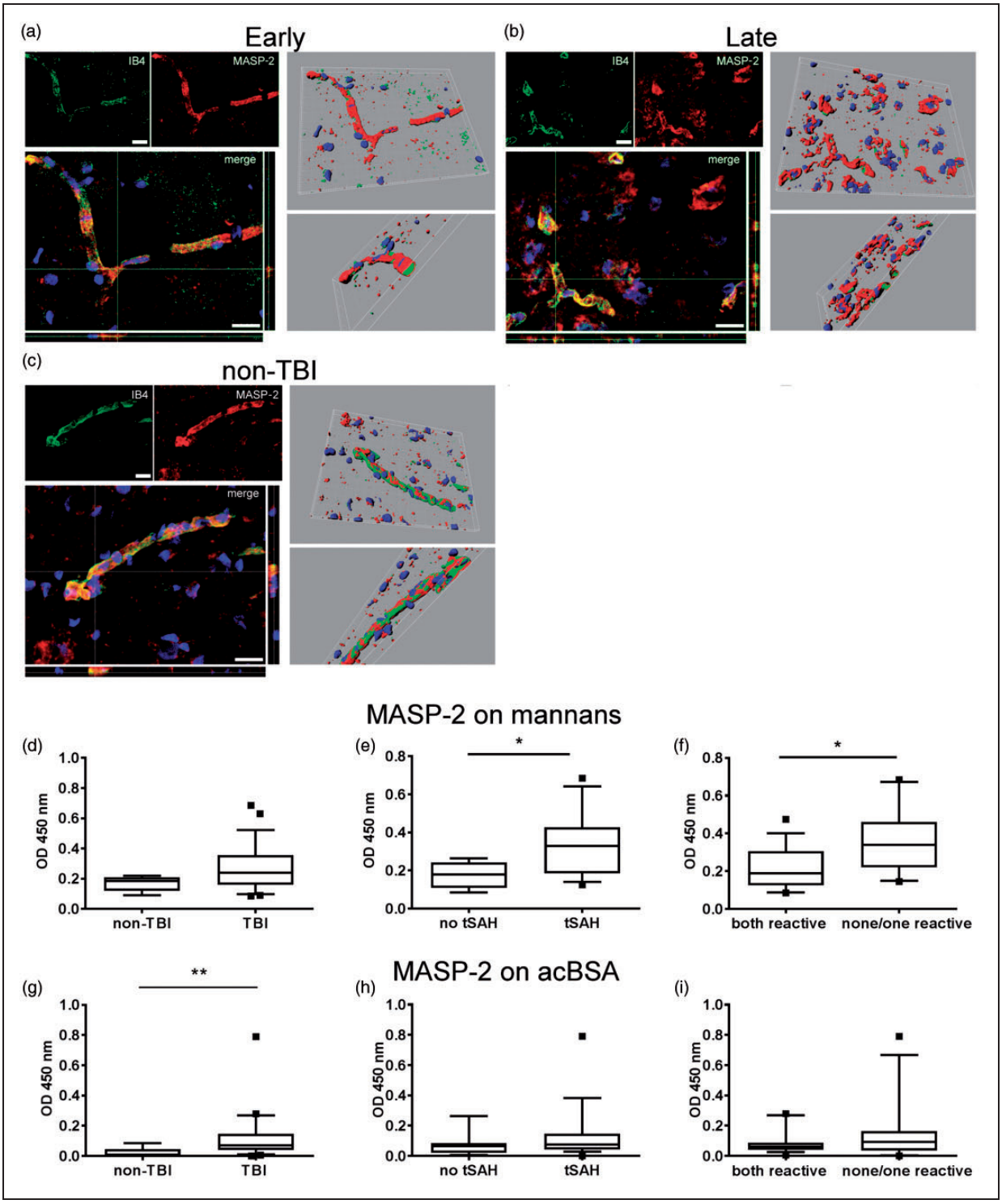

Figure 4. MASP-2 is higher in TBI than non-TBI human cerebral tissues and its levels are associated with more severe clinical conditions. Representative images of MASP-2 (*red*), vessels (IB4, *green*) and nuclei (Hoechst, *blue*) in early and late removed contusions (a, b) and in non-TBI cerebral tissues (c). Single xy plane views with z projection (merge images) and 3D renderings demonstrate MASP-2 in the surroundings of cerebral vessels and in cerebral parenchyma both in early (a) and late (b) contusions. It can be detected near cerebral vessels also in non-TBI (c) cerebral tissues. Images are representative of at least two specimens per group. Scale bars 20 μm, thicks in 3D renderings 10 μm. MBL-mediated MASP-2 deposition (MASP-2 on mannans) showed a tendency toward an increase in TBI specimens compared to non-TBI ones (non-TBI: $n = 5$, TBI: $n = 28$, d). It was significantly higher in homogenates from patients with tSAH (no tSAH: $n = 11$, tSAH: $n = 17$, e) and/or none/one reactive pupils (both reactive: $n = 17$, none/one reactive: $n = 11$, f). Ficolin-mediated MASP-2 deposition (MASP-2 on AcBSA) was significantly higher in TBI than non-TBI specimens (non-TBI: $n = 5$, TBI: $n = 28$, g). No difference was found on stratifying TBI patients for tSAH (no tSAH: $n = 11$, tSAH: $n = 17$, h) and/or pupil reactivity (both reactive: $n = 17$, none/one reactive: $n = 11$, i). Data are reported as box plots and 10th and 90th percentiles. Mann–Whitney test: *$p < 0.05$; **$p < 0.01$.

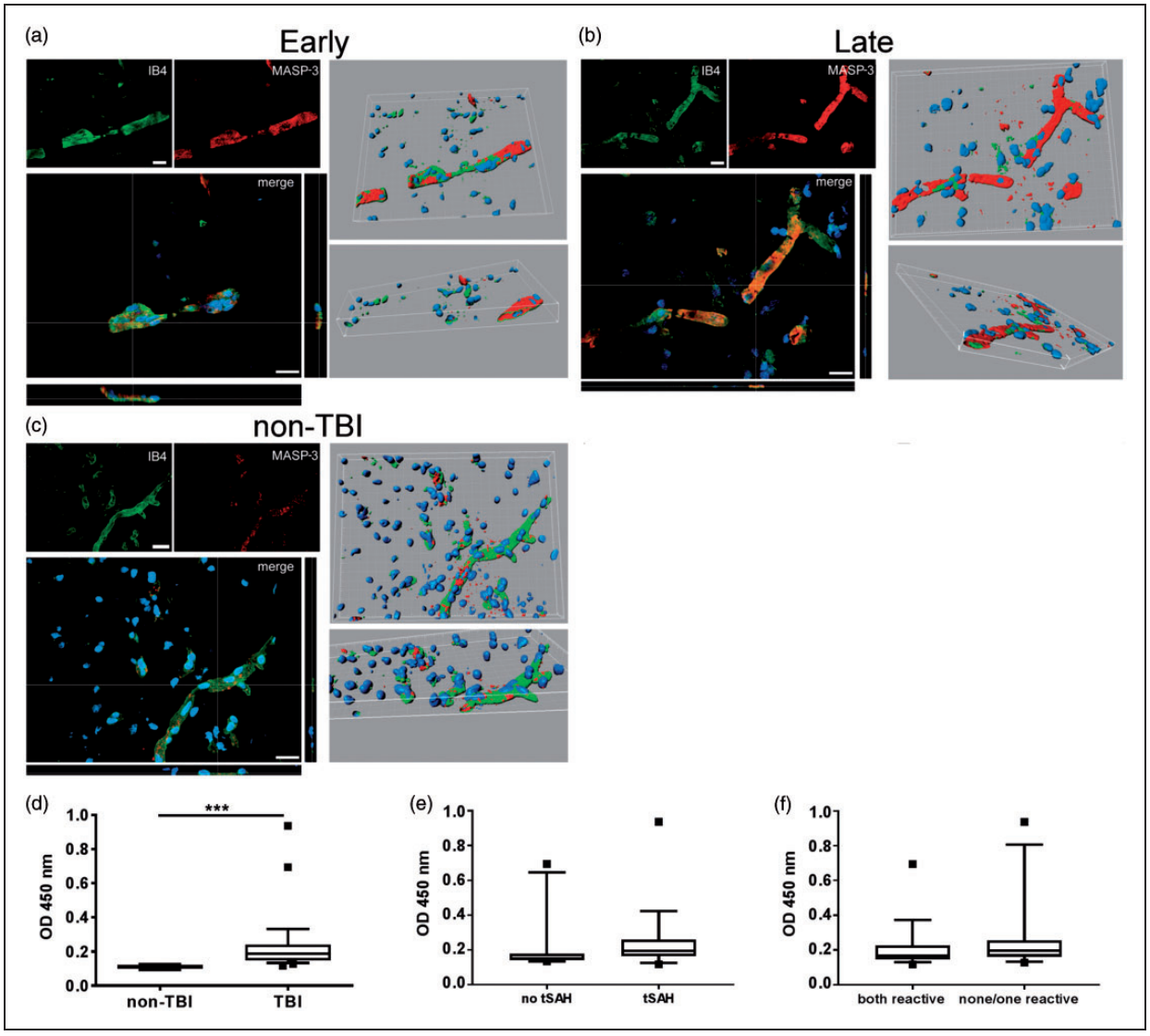

Figure 5. MASP-3 is higher in TBI than in non-TBI human cerebral tissues. Representative images of MASP-3 (*red*), vessels (IB4, *green*) and nuclei (Hoechst, *blue*) in early and late removed contusions (a, b) and in non-TBI cerebral tissue (c). Single xy plane views with z projection (merge images) and 3D renderings indicate that MASP-3 is present in cerebral vessels both in early (a) and late (b) contusions. A weak positive signal was detected also in non-TBI (c) cerebral tissues. Images are representative of at least two specimens per group. Scale bars 20 μm, thicks in 3D renderings 10 μm. Homogenates from contusions had higher MASP-3 levels than non-TBI tissues (non-TBI: $n = 4$, TBI: $n = 28$, d). Data are reported as box plots and 10th and 90th percentiles. Mann–Whitney: ***$p < 0.001$. No difference was found on stratifying TBI patients for tSAH (no tSAH: $n = 11$, tSAH: $n = 17$, e) and/or pupil reactivity (both reactive: $n = 17$, none/one reactive: $n = 11$, f). Data are reported as box plots and 10th and 90th percentiles. Mann–Whitney test: ns.

alternative pathway; (2) the lectin pathway components are persistently present, up to five days post-TBI; (3) the levels of MASP-2, a key enzyme driving lectin pathway activation, are increased and significantly associated with TBI severity.

This study stems from our previous findings that the lectin pathway of complement system activation is implicated in experimental TBI and that its functional inhibition is protective.[13,14] In fact mice genetically deficient for MBL, one of the activators of the lectin pathway, show attenuated sensorimotor deficits after TBI.[14] In addition, administration of a polymannosylated compound, which inhibits MBL, has similar protective properties thus indicating that the lectin pathway is potentially eligible for drug targeting in TBI.[13]

In clinical TBI, circulating lectin pathway initiators appear to be associated with injury severity and can predict unfavorable outcome in patients.[21,29] Thus, high serum levels of MBL or low serum levels of ficolin-3 appear to be associated with injury severity and

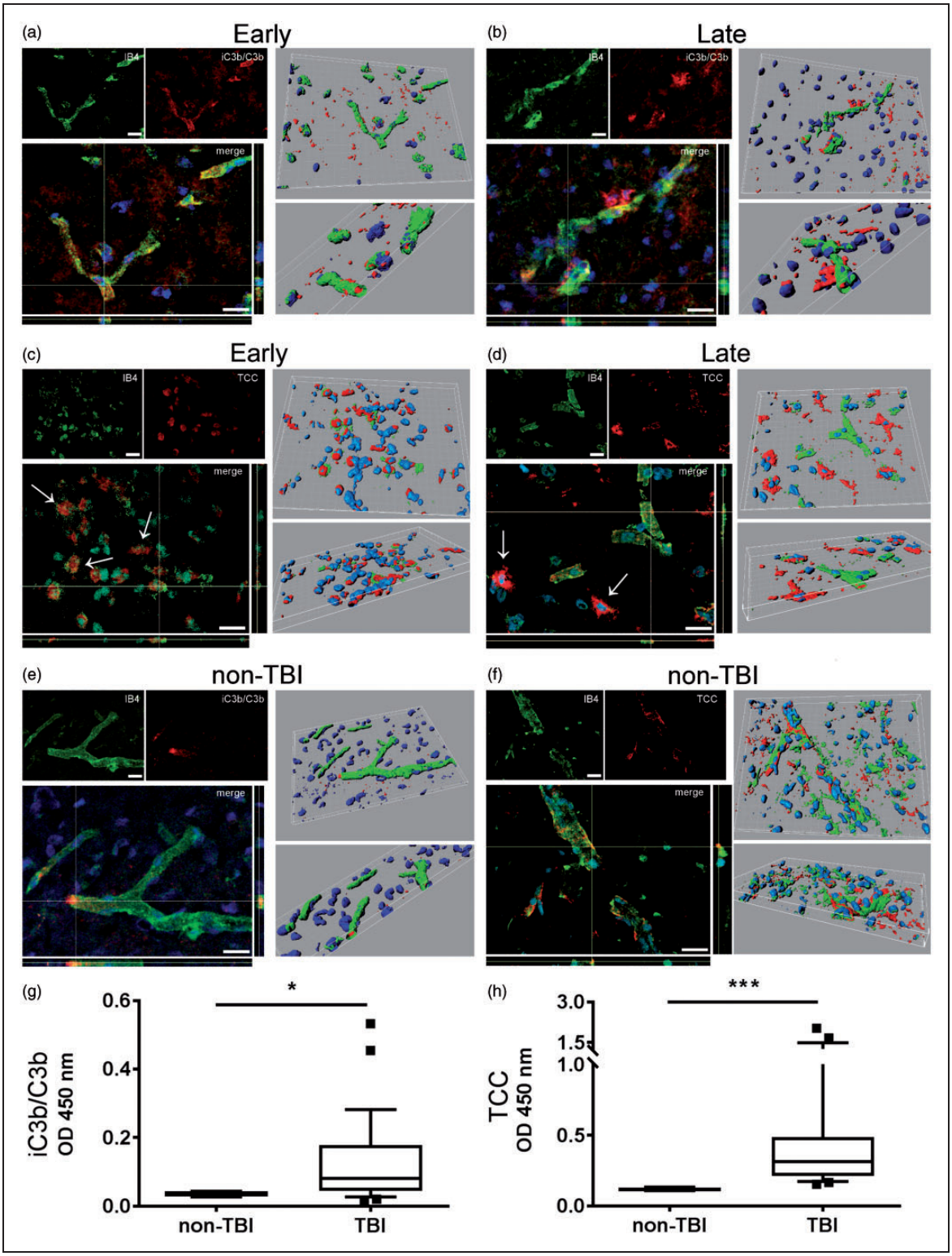

Figure 6. C3 active fragments and TCC are present and increased in TBI human contusions. Representative images of C3 active fragments (iC3b/C3b, *red*) or terminal complement complex (TCC, *red*), vessels (IB4, *green*) and nuclei (Hoechst, *blue*) in the contusional tissues removed early (a, c) or late (b, d) after TBI and in non-TBI tissues (e, f). Single xy plane views with z projections (merge images) and 3D renderings are presented. C3 active fragments are present inside and outside cerebral vessels both in early (a) and late (b) samples, while they are located mainly inside cerebral vessels and are weaker in non-TBI brain tissue (e). TCC is present extravascularly in TBI contused tissues where it localizes around cell bodies (white arrows in c, d), which are not observed in non-TBI tissues (f). Images are representative of at least two specimens per group. Scale bars 20 μm, thicks in 3D renderings 10 μm. Homogenates from contusions had higher levels of C3 fragments and TCC than non-TBI brain tissues (non-TBI: $n = 4$, TBI: $n = 28$, g, h). Data are reported as box plots and 10th and 90th percentiles. Mann–Whitney: $^{*}p < 0.05$; $^{***}p < 0.001$.

act as independent predictors of outcome. However, since circulating complement components may be subject to rapid turnover and consumption due to the activation of pathway, affecting the overall circulating concentrations, there is still no clear picture on the role of the lectin pathway in clinical TBI. Here we report MBL in TBI contusions – with no difference between those removed within 12 h or from >12 h until five days after TBI – thus expanding our previous observations.[14] In addition, for the first time we document the presence of ficolin-1, ficolin-2 and ficolin-3 in contused brains. Unlike MBL whose presence is limited to TBI contusions, ficolins are also seen in non-TBI specimens. Ficolin-1 can be clearly observed near the vasculature and in brain parenchyma, while ficolin-2 and -3 are scarcely present and mostly located in proximity of brain vessels. Again, unlike MBL, ficolins appear to extravasate more than MBL in TBI patients. This different behavior may possibly be due to: (1) MBL's ability to bind to epitopes expressed on activated endothelial cells which are not recognized by ficolins; (2) larger MBL circulating complexes which thus have less access to the brain parenchyma through the injured BBB; (3) lower circulating levels of MBL than ficolins. Overall, all lectin pathway initiators appear to be present in the brain parenchyma in the vicinity of vessels thus suggesting that the blood compartment is a major source, although a contribution of direct brain cell synthesis cannot be excluded.

Quantification of the levels of lectin pathway activators in contusion homogenates showed that MBL, ficolin-2 and ficolin-3 are significantly higher in TBI patients than individuals with no TBI. The lower ficolin-3 circulating levels after TBI reported by Pan et al.[29] may depend on protein consumption due to brain accumulation, as shown here, contributing to full complement activation at the site of cerebral contusion, as indicated by the central presence of iC3b/C3b and TCC. Unlike the other initiators, ficolin-1 does not appear to be a specific marker for TBI. Differently from ficolin-2 and -3 that are mainly produced by the liver and lung and circulate in the bloodstream, ficolin-1 is primarily synthesized and presented on the surface of circulating monocytes and neutrophils, promoting their adhesion, aggregation and migration.[22] Thus, the ficolin-1 in our specimens may be triggered by inflammatory mechanisms related to immune cell recruitment and leukocyte activation, similarly to what has been reported in stroke patients.[22]

Lectin pathway initiators circulate in blood associated with serine proteases (MASPs). On binding their targets, MASPs become activated, promoting downstream complement activation. Of the three known MASPs, MASP-2 is the key enzyme driving lectin pathway activation in acute brain injury.[40] MASP-2 is present in TBI specimens, where it appears to be located both in parenchyma and near the vessels, as well as in non-TBI specimens, where its location is mainly on vessels. MASP-2 tissue distribution mirrors that of C3 fragments and TCC, suggesting that local complement activation comes from the lectin pathway. Using ELISA to measure MASP-2 deposition on mannans (the MBL preferred ligand) or on acBSA (the ficolins' preferred ligand), we show that ficolin-driven MASP-2 deposition is significantly higher in TBI vs. non-TBI homogenates. Importantly, MBL-driven MASP-2 deposition is increased in TBI patients with more severe trauma, indicated by abnormal pupil reactivity and/or traumatic SAH.[42,43] These factors are strong predictors of worse outcome in a well-validated prognostic model.[43,44] We did not detect any significant association between lectin pathway protein cerebral levels and outcome six months after TBI. This might be due to the limited number of cases with favorable outcome, which can be explained by a possible selection bias. In fact, we included only patients with large mass lesions and related extended brain injury, needing lifesaving neurosurgery. The cohort analyzed also had a high median age (58) and rate of pupillary abnormality (39%) – two strong predictors of poor outcome.[43,44] In fact, 78% of our patients had an unfavorable outcome (GOS: 1–3) six months after TBI. A recent study by Osthoff et al.[45] on a younger and less severe cohort of TBI patients reported that circulating MASP-2 levels were associated with poor outcome at 90 days, thus lending further support to the hypothesis of the lectin pathway, particular by MASP-2, as involved in the pathology. The molecular basis of the detrimental effects of the MBL:MASP-2 complex still needs to be fully elucidated. Data in experimental models of stroke helped identify several downstream vascular effects associated with the complex activation.[46] Similar mechanisms might be involved in the traumatic pericore tissue, an area subjected to post-injury hypoxia.[47]

The exact physiological role of MASP-3 is still largely unknown. This protease circulates in association with lectin pathway initiators, but is required for alternative pathway activation, acting as a major activator of pro-FD.[48,49] We report high levels of MASP-3 in TBI compared to non-TBI homogenates, implying a possible role for the alternative pathway too in TBI pathophysiology, as reported in experimental models.[50,51]

Downstream in the complement cascade, C3 convertase cleaves C3, forming C3 active fragments. Previous studies reported higher serum and cerebral spinal fluid levels of total C3 (complete protein and fragments) in TBI than in non-TBI patients.[52,53] C3 fragments were reported on presumed neuronal cell surfaces in human contusions.[6] Here, using an antibody specific for C3 activation fragments, we found iC3b/C3b fragments

in TBI contusions located both close to brain vessels and in the parenchyma, and more than non-TBI specimens. Immediately after the impact and persistently up to five days, C3 products are present, and may opsonize cells and trigger the subsequent phagocytosis of damaged cells. Although local C3 synthesis may contribute to the presence of C3 in the brain tissue, C3 cleavage products may rapidly gain access to the brain parenchyma through a damaged BBB. In non-TBI specimens, where the BBB is expected to be intact, iC3b/C3b fragments are located only inside cerebral vessels, and to a lesser extent than in contused cerebral tissues.

The formation of TCC, the complex that damages cell membranes causing their final lysis, is the final step of the complement cascade. An early study by Bellander et al.[6] reported increased immunoreactivity for TCC in contused human tissues. Using immunoassay and confocal analysis, we found higher levels of TCC in cerebral homogenates from TBI compared to non-TBI patients. No difference in TCC staining was found between human contusions removed early or late after TBI, indicating strong and persistent activation of the terminal pathway of the complement cascade after the injury. TCC appeared to surround cellular bodies, possibly neurons, one of the main targets of complement after TBI.[6]

The non-TBI group included a sample with outlier values for iC3b/C3b and TCC levels which were excluded from the statistical analysis. Interestingly, this was a tumor biopsy (patient: Tumor 2) which had low MASP-2 and high MASP-3 levels, in line with high expression of alternative pathway components and specific induction of the alternative pathway reported in glioma cell lines.[54,55] The non-TBI controls might fail to represent the healthy population, but they allowed us to define a specific pattern of complement activation in TBI. A possible limitation of using non-TBI autoptic tissues is the occurrence of post mortem autolysis. However, the neuronal structures (targets of iC3b/C3b and TCC) are known to be preserved till 48 h post mortem.[56]

Clinical and experimental data indicate that the complement system is implicated in post-injury inflammation and neuropathology after TBI.[6–10,14,15,17] Of the three activation pathways, there is no evidence for a role of the classical pathway,[17] while the alternative one, in addition to the lectin pathway, may also be involved in TBI neuropathology. This study documented the presence of the lectin pathway components in human cerebral contused tissue. Once in the brain parenchyma, the lectin pathway drives full complement activation which may lead to neuroinflammation and tissue injury.[27,46,57] Since the lectin pathway after TBI is associated with injury severity, is persistent and druggable – as indicated by experimental data[13,20] – this offers an opportunity for the development of pharmacological interventions.

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Declaration of conflicting interests

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Authors' contributions

DDB, SF, LN conducted the experiments, acquired and analyzed the data, wrote the ms; CP, FaO, FrO acquired and analyzed the data; ERZ, EP, ML, NS, LL provided human specimens, acquired the data; PG acquired and analyzed the data, provided reagents; MGDS designed the study, analyzed the data, wrote the ms

Supplementary material

Supplementary material for this paper can be found at the journal website: http://journals.sagepub.com/home/jcb

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