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Inhibition of the Membrane Attack Complex of the Complement System Reduces Secondary Neuroaxonal Loss and Promotes Neurologic Recovery after Traumatic Brain Injury in Mice

Kees Fluiter,* Anne Loes Opperhuizen,* B. Paul Morgan,† Frank Baas,* and Valeria Ramaglia*

Traumatic brain injury (TBI) is the leading cause of disability and death in young adults. The secondary neuroinflammation and neuronal damage that follows the primary mechanical injury is an important cause of disability in affected people. The membrane attack complex (MAC) of the complement system is detected in the traumatized brain early after TBI; however, its role in the pathology and neurologic outcome of TBI has not yet been investigated. We generated a C6 antisense oligonucleotide that blocks MAC formation by inhibiting C6, and we compared its therapeutic effect to that of Ornithodoros moubata complement inhibitor (OmCl), a known inhibitor of C5 activation that blocks generation of the anaphylatoxin C5α and C5b, an essential component of MAC. Severe closed head injury in mice induced abundant MAC deposition in the brain. Treatment with C6 antisense reduced C6 synthesis (85%) and serum levels (90%), and inhibited MAC deposition in the injured brain (91–96%). Treatment also reduced accumulation of microglia/macrophages (50–88%), neuronal apoptosis, axonal loss and weight loss (54–93%), and enhanced neurologic performance (84–92%) compared with placebo-treated controls after injury. These data provide the first evidence, to our knowledge, that inhibition of MAC formation in otherwise complement-sufficient animals reduces neuropathology and promotes neurologic recovery after TBI. Given the importance of maintaining a functional complement opsonization system to fight infections, a critical complication in TBI patients, inhibition of the MAC should be considered to reduce posttraumatic neurologic damage. This work identifies a novel therapeutic target for TBI and will guide the development of new therapy for patients. The Journal of Immunology, 2014, 192: 2339–2348.

Studies of traumatic brain injury (TBI) in human and experimental models have identified the complement system as an early mediator of posttraumatic neuroinflammation and secondary neuronal damage, ultimately leading to behavioral, emotional, and cognitive problems (1–8). Therefore, timely intervention to target activation of the complement system may be a promising therapy to reduce neuropathology and improve neurologic outcome in patients with TBI.

Therapies aimed to inhibit complement activation have proved to be protective in experimental models of TBI; however, most therapeutic strategies targeted the complement cascade at its core, the C3 convertase, resulting in a general and broad inhibition of the complement system (9, 10). Alternative strategies have targeted the anaphylatoxin C5α, a potent inflammatory mediator of the complement cascade (11), whereas other approaches interfered with activation of the alternative pathway of complement (12), known to play a major role in the pathophysiology of many diseases (13). Given the importance of maintaining a functional opsonic complement system to mediate an effective immune response against infections, a critical complication in TBI patients (14), we considered the possibility that inhibition of the most downstream component of the terminal complement activation pathway, formation of the membrane attack complex (MAC), would be sufficient to prevent secondary neurologic damage and neurologic deficit after TBI.

We have previously shown that the MAC attacks peripheral nerves damaged by a traumatic injury, exacerbates posttraumatic damage, and impairs recovery of function (15–17). Another study showed that deletion of C6, a necessary component of the MAC, is neuroprotective after peripheral nerve trauma (15, 17). Another study showed that deletion of CD59a, the major regulator of MAC formation in mice, aggravates secondary neuronal cell death after brain trauma, suggesting a key role for MAC in the pathophysiology of TBI (8). However, the effects of specific inhibition of MAC formation have never been tested in TBI.

In this article, we implemented a closed head injury model of TBI in mice (18) to determine the extent of MAC deposition in the brain after mild or severe injury. In addition, we tested the effects on the outcome of severe TBI of MAC inhibition using either a C6 antisense oligonucleotide, which prevents MAC formation by blocking C6 protein synthesis, or the C5-binding protein Ornithodoros moubata complement inhibitor (OmCl), which blocks generation of both C5α and MAC (19). Data presented in this article show that inhibition of MAC formation is sufficient to...
prevent secondary neuropathy and improve neurologic performance after severe TBI. These findings will help guide the development of novel therapies for people with TBI.

Materials and Methods

Animals

Male 8-wk-old C57BL/6 (n = 69) or female 8-wk-old BALB/c (n = 61) mice were obtained from Harlan (Bicester, U.K.) and housed in groups for at least 1 wk before the start of the experiments. Animals weighed, on average, 25.36 ± 1.24 g at the beginning of the study and were given food and water ad libitum. They were kept at room temperature on a 12-h light/dark cycle. All experiments were approved by the Academic Medical Center Animal Ethics Committee and complied with Dutch national guidelines for the care of experimental animals.

Closed head injury model

Focal brain injury was induced by a standardized free-falling weight drop device, reproduced from Flierl et al. (18). In brief, the device uses a free-falling rod weighing 340 g. The falling height is adjusted to determine the degree of injury. The rod is held by a magnetic locking mechanism controlled by a pedal. Pressing the pedal releases the rod and causes a free-fall. The device ends with a blunt tip of 3 mm in diameter, which impacts the exposed skull of the mouse, immobilized on a mobile platform at the bottom of the device. The trauma was induced under deep isoflurane anesthesia (1.5 v/v isoflurane and 1.0 v/v O2).

Five minutes before the trauma, mice were treated s.c. with one dose (0.05 mg/kg) of buprenorphine (Temgesic, Schering-Plough, The Netherlands) as analgesic. After hair removal, the skull was exposed by a midline incision. In the initial experiment, mice were subjected to a weight drop of 1 (n = 3), 2 (n = 7), or 3 (n = 3) cm. All subsequent experiments were performed using a 2-cm falling height (n = 53), referred to in the text as severe TBI. After trauma, the scalp was closed with small clamps and mice were placed on a 37˚C pad to recover from anesthesia before being returned to their home cages. All TBIs were performed in the morning.

Neurologic severity score and weight

To evaluate neurologic impairment, we scored all mice on a 10-item rating performance, which tests the function of the cortex, the midbrain, and the cerebellum, including motor function, balance, alertness, and physiologic behavior. In brief, mice were tested for: 1) the ability and initiative to exit a circle of 30-cm diameter within 3 min; 2) paresis of upper and/or lower limb of the contralateral side; 3) alertness, initiative, and motor ability to walk straight; 4) a reflex: the mouse will bounce in response to a loud hand clap; 5) physiologic behavior as a sign of “interest” in the environment; 6) ability to balance on a beam of 7-mm width for at least 10 s; 7) ability to balance on a round stick of 5-mm diameter for at least 10 s; 8) ability to cross a 30-cm-long beam of 2-cm width; 9) ability to cross a 30-cm-long beam of 7-mm width; 10) ability to cross a 30-cm-long beam of 5-mm width. Each task is scored 0 for success or 1 for failure. The sum of the scores from the 10 tasks is the neurologic severity score (NSS) (18). NSS was assessed at 1, 4, 24, 48, and 72 h post-TBI. In addition, all mice were weighed immediately before trauma and every day afterward.

Generation of MAC inhibitors

The C6 Locked Nucleic Acid (LNA) oligonucleotides were synthesized with phosphorothioate backbones and methylated DNA–C (medC) by Ribotask (Odense, Denmark) on a Mermade 12, using 2 g NitoPhase (BioAutomation). All fractions were analyzed by IEX-HPLC on a Merck Hitachi D-7000 HPLC system using a DNA Pac PA100 (4 × 250 mm) column (Dionex). The fractions containing the pure oligonucleotide were pooled and desalted (until conductivity <1.0 mS/cm) on an Aktac Cross-Flow (GE Healthcare) equipped with a Pellicon 2 filter (type: PLAC, area: 0.1 m2 from Millipore). Throughout the process, the oligonucleotide constitution was confirmed by MALDI-TOF mass spectrometry analysis on a BB-Bruker Autospec using 3-hydroxy-3-methylglutaryl acid as matrix substance. The sequence of the C6 antisense oligonucleotide is 5′-AACttgctgggAgAAT-3′. LNA are shown in capital letters and DNA in lowercase. The tick-derived sequence of the C6 antisense oligonucleotide is 5′-9acctttacctcggcaagtttct-3′. The reaction in the different organs was corrected for serum present at the time of sampling as determined by the distribution of [125I]-labeled BSA.

In situ detection of the MAC inhibitor

In situ detection of C6 antisense oligonucleotide was done by hybridizing a full LNA FAM-labeled sense probe (5′-FAM-GCCCTTTAGA-3′) against the C6 antisense oligo on paraffin-embedded liver tissue. Livers of mice treated with C6 antisense oligonucleotides were fixed in formalin for standard processing into paraffin. Sections of 5-μm thickness were deparaffinized and boiled for 5 min in 0.1 M citric acid, pH 6. Hybridization was done for 1 h at 60˚C in hybridization mixture containing 0.5 μM, 500 nM NucCl, 10 mM TEPS buffer, pH 7.5, 1 mM EDTA, 5% Denhardt’s reagent. Probe concentration was 50 nM. Detection was done using anti-fluorescein FAB fragments labeled with alkaline phosphatase (Roche) 1:1000 and Vector Alkaline Phosphatase Substrate kit III (Vector).

Optimization and dosing of the MAC inhibitor

C6 antisense oligonucleotides (LNA in capital letters, DNA in lowercase letters) were 14 mer, GTGttattcaAG/12 mer, GTGttatCAA or 11 mer, GTGttatCA. The oligonucleotides were dissolved in PBS and injected once daily for 3 d in female BALB/c mice at defined doses (0.5, 1, and 5 mg/kg; 3 mice per oligonucleotide per dose). Mice were sacrificed 4 d after the last injection.

ELISA

Mouse blood was collected by venipuncture from C6 antisense-treated or PBS-treated control mice at defined time points after the end of the treatment. After clotting on ice, the serum was separated by centrifugation at 5000 × g at 4˚C for 10 min and assayed immediately. ELISA to detect phosphorylated neurofilament H chain (pNF-H) in mouse serum was performed using a kit according to manufacturer’s instructions (Biovend, Oxford, U.K.). ELISA to detect C6 in mouse serum was performed according to a standard protocol using the in-house developed polyclonal rabbit anti-mouse C6 Ab (generous gift from Prof. Moh Daha).

Quantitative PCR

Total RNA was extracted from liver tissue of C6 antisense-treated mice or antisense mismatch- or PBS-treated controls using TRIZol (Invitrogen, Germany) and chloroform for phase separation and isopropanol precipitation. For the reverse transcription reaction, 0.5 μg RNA was mixed with 125 pmol/μl OligoDT12-2VN and denatured for 10 min at 70˚C. cDNA was synthesized using SuperScriptII reverse transcriptase (Invitrogen) and incubating at 42˚C for 1 h. Quantification of C6 and C3 mRNA was done on a LightCycler 480 (Roche) with the Universal probe system (Roche). Hypoxanthine phosphoribosyl transferase was used as reference gene. The following primers were used (Sigma-Aldrich): C6 mouse F′ 5′-cagaaagaagacctccatta-3′, C6 mouse R′ 5′-ttctggaggaagttaaatgac-3′; for C3: C3 mouse F′ 5′-accttactcgccaggttct-3′, C3 mouse R′ 5′-tgtagcctggcttag-3′; for HPRT, HPRT rat/mouse F′ 5′-gctttcaggctactgtag-3′, HPRT rat/mouse R′ 5′-caactagcctttgactc-3′; Universal Probe #13 (Roche reference no. 04685121001) for C6; Universal Probe #76 (Roche reference no. 04688996001) for C3; Universal Probe #22 (Roche reference no. 04688060001) for HPRT. Quantitative PCR (qPCR) was done according to manufacturer’s instructions (Roche). At least two independent experiments in triplicate were performed for each cDNA tested. Data were analyzed using the advanced relative quantification module in the LightCycler 480 software (Roche).

Treatment with inhibitors of the terminal complement pathway

Inhibition of MAC formation in mice was achieved either by antisense targeting C6 mRNA to prevent synthesis of C6 or by targeting C5 protein with OmCl. Both block assembly of the MAC and the latter also prevents formation of the anaphylatoxin C5a. To inhibit C6 synthesis, we treated mice (n = 9) with the selected C6 antisense oligonucleotide (14 mer, 5 mg/kg s.c.) for 4 d, starting at 6 d before TBI to allow sufficient time for downregulation of C6 protein levels. To inhibit C5 cleavage, we treated mice with a bolus i.v. injection of OmCl given either immediately before TBI (n = 10) or 15 min post-TBI (n = 13) or 30 min post-TBI (n = 4). The dose of 5 mg/kg and the i.v. route of administration for OmCl was previously shown to produce complete inhibition of serum hemolytic activity within 5 min from administration in mice, with a half-life of 12 h (19). Placebo control mice were injected with equal volume of PBS. Sham-operated mice, which underwent the same surgical procedure, anesthesia, and analgesia but did not receive TBI, were used as additional control.
Tissue processing
At 72 h post-TBI, mice were anesthetized and intracardially perfused with PBS followed by 4% paraformaldehyde. Blood was collected to measure serum levels of pNF-H or C6. Liver from C6 or mismatch antisense-treated or PBS-treated mice was collected and immediately placed into RNA later, according to manufacturer’s instructions (Ambion, Bleiswijk, The Netherlands), to determine C6 or C3 mRNA levels. Brains were dissected out and placed in formalin for standard processing into paraffin and used for immunohistochemical studies.

Immunohistochemistry
Left and right brain hemispheres were embedded into paraffin blocks. Sagittal sections of 6 μm thickness were cut, stretched in a 40°C water bath, and mounted on glass slides. Sections were deparaffinized in a standard series of xylene and alcohol. Endogenous peroxidase was blocked with 0.3% H2O2 in methanol for 20 min at RT. Ag retrieval was performed in the relevant buffer (see Table I) by microwave for 13 min at full power. After cooling down, sections were incubated with 10% normal goat serum in PBS to block nonspecific binding sites. Sections were then incubated with the primary Ab (see Table I) for 1 h at RT or overnight at 4°C. All sections were incubated with HRP-linked secondary Ab for 30 min at RT. Peroxidase activity was visualized by 3,3-diaminobenzidine tetrahydrochloride (1:10 in 0.05 M Tris-HCl pH 7.6). Sections were counterstained with hematoxylin for 5 min followed by running water for 4 min. After a series of dehydration steps in alcohol and xylene, sections were mounted in mounting medium (Pertex; Histolab, Gothenburg, Sweden) and allowed to dry in air.

Quantification of immunohistochemistry
Images (5–10 per brain area) of immunostainings for C9, Iba-1, and CD11b covering >90% of the cerebellum, hippocampus, periventricular, and subpial area of mouse brain sections (3–4 per mouse) were captured with Olympus BX41 microscope and processed with Cell-D software. The percentage of staining per unit area was quantified using Image Pro Plus 7.0 analysis software.

Statistical analysis
All statistical analysis was performed using GraphPad Prism 7.0 analysis software. Two-way ANOVA analysis, including Bonferroni correction and post hoc tests, was used for statistical analysis of the NSS, weight loss, and the quantification of the immunohistochemical stainings. One-way ANOVA analysis, including Bonferroni correction, was used for the statistical analysis of qPCR, ELISA, and hemolytic assay for the C6 antisense characterization experiments. A t test was used for statistical analysis of the qPCR results for the C6 antisense therapy experiment. Differences were considered statistically significant when \( p < 0.05 \). All values are given as means ± SEM.

Results
Severe closed head injury induces neurologic disability, neuroaxonal damage, and edema
To mimic TBI, we implemented a mouse model of closed head injury as described by Flierl et al. (18). The height of the weight-drop device was adjusted to produce either a mild or severe injury. A weight drop of 3 cm was fatal for all mice tested. A weight drop of 2 cm produced 20% mortality rate, whereas all mice survived the 1-cm drop. The NSS of mice hit by the 2-cm drop showed maximal neurologic disability (NSS of 10) at 1 h after TBI, decreasing to NSS of 2.0 ± 1.0 at 72 h after TBI. Mice hit by the 1-cm drop scored a significantly lower NSS (6.0 ± 2.1) at 1 h after TBI compared with the 2-cm–injured mice (\( p < 0.05 \), two-way ANOVA), reaching complete recovery after 48 h (\( p < 0.05 \), two-way ANOVA compared with 2-cm drop; Fig. 1A).

pNF-H, a major component of neurons and axons, accumulates in the blood after brain damage. Therefore, pNF-H serum levels are commonly used as an indirect marker of neuroaxonal injury (22). The 2-cm drop produced a significant increase in pNF-H serum levels from a basal level of 134.3 ± 3.7 to 4327.0 ± 792.2 pg/ml (\( p < 0.05 \), two-way ANOVA) at 72 h postinjury, whereas the pNF-H serum levels of mice hit by the 1-cm drop did not differ significantly from control levels at 72 h postinjury (Fig. 1B).

Macroscopic examination of the brains at 72 h after TBI (Fig. 1C–E) showed edema and hemorrhage at the site of impact in mice hit by the 2-cm drop (Fig. 1E, arrow). Albumin immunoreactivity (Fig. 1F–H and see Table I) confirmed extravasation of fluids into the brain parenchyma in these mice (Fig. 1H, arrows), whereas the 1-cm drop did not produce edema or evident blood–brain barrier (BBB) leakage (Fig. 1D, arrow, 1G).

Taken together, these findings indicate that the 2-cm drop caused severe neurologic disability, neuroaxonal damage, and BBB leakage compared with the 1-cm drop, which caused only mild, transient neurologic impairment.

Severe closed head injury induces MAC deposition and inflammation at 72 h postinjury
To determine whether TBI induces MAC deposition and inflammatory changes in the injured brain, immunostainings for C9, Iba-1, and CD11b (see Table I), were performed on sagittal sections of the brain from mice hit by either a 1- or 2-cm drop or uninjured mice.

Immunoreactivity for C9 (Fig. 2A–C), with an Ab that in activated mouse serum specifically recognizes native C9 and C9 complexed into the soluble C5b-9 complex or terminal complement complex (Supplemental Fig. 1) and in fixed tissue is a marker for MAC deposition (15, 23–25), was abundant in the brains of mice hit by the 2-cm drop (Fig. 2C). Quantification of the C9 staining showed significantly higher amounts of C9 immunoreactive area in the cerebellum, hippocampus, periventricular area, and subpial area of the brain hit by the 2-cm drop (3- to 4-fold increased in all areas, \( p < 0.01 \), two-way ANOVA), MAC immunoreactivity was virtually undetectable in uninjured mice (Fig. 2D).

Immunoreactivity for Iba-1 (Fig. 2E–G), a marker of microglia/macrophages, showed enlarged microglia/macrophase morphology, typical of activated cells, in the brain of mice hit by the 2-cm drop (Fig. 2G). Quantification of the Iba-1 staining showed a significantly higher amount of Iba-1 immunoreactive area in all four examined brain areas from mice hit by the 2-cm drop compared with the 1-cm drop (2- to 3.5-fold increased, \( p < 0.01 \), two-way ANOVA).
ANOVA) and uninjured brains (4.5- to 21-fold increased, \( p < 0.01 \), two-way ANOVA; Fig. 2H).

Immunoactivity for CD11b (Fig. 2I–K), a marker of microglia/macrophages and whose expression increases on activation, was

**FIGURE 1.** Evaluation of posttraumatic neurologic impairment, neuroaxonal damage, and edema. (A) NSS determined in mice at 1, 4, 24, 48, and 72 h after TBI induced by either 1- (\( n = 3 \)) or 2-cm (\( n = 7 \)) drop. A score of 10 means that all tasks are failed; a score of 0 means that all tasks are successfully completed. (B) Levels of pNF-H (measure of neuroaxonal damage) quantified by ELISA in serum from uninjured mice and mice injured by either 1- or 2-cm drop at 72 h after TBI. (C–E) Macroscopic images of brains from uninjured mice or mice hit by either 1- or 2-cm drop at 72 h after TBI, showing edema and hemorrhage at the impact site after the 2-cm drop (E, arrow), but not after the 1-cm drop (D, arrow). (F–H) Immunostaining for albumin in sagittal sections of the cerebellum showing immunoreactivity within the parenchyma in mice hit by 2-cm drop (H, arrows and inset), indicating extravasation of fluids from the circulation. Albumin immunoreactivity is not detected in the cerebellum of mice hit by 1-cm drop (G) or uninjured mice (\( n = 3 \)) (F). Asterisks indicate statistically significant differences compared with uninjured controls, *\( p < 0.05 \).

**FIGURE 2.** MAC deposition and inflammation at 72 h after TBI. Representative images of immunostainings for MAC with the anti-C9 Ab (A–C), pan microglia/macrophages with the anti-Iba-1 Ab (E–G), and activated microglia/macrophages with the anti-CD11b Ab (I–K), showing intense MAC immunoreactivity, enlarged microglia/macrophages morphology (sign of active phenotype), and evident CD11b immunoreactivity on the membranes of macrophages (sign of increased activity) in the parenchyma of the cerebellum of mice hit by 2-cm drop (\( n = 7 \)) (C, G, and K, respectively). Quantification of immunostainings for C9 (D), Iba-1 (H), and CD11b (L) in cerebellum (cb), hippocampus (hc), periventricular area (pv), and subpial area (sp) of mice hit by either 1- (\( n = 3 \)) or 2-cm (\( n = 7 \)) drop compared with uninjured mice (\( n = 3 \)). Asterisks indicate statistically significant differences compared with uninjured controls: *\( p < 0.05 \), **\( p < 0.01 \).
consistently detected in the brain of mice hit by the 2-cm drop (Fig. 2K). Quantification of the CD11b staining showed significantly higher amounts of CD11b immunoreactive area in the cerebellum, periventricular, and subpial areas of brains hit by the 2-cm drop compared with the 1-cm drop (up to 2-fold increased in all areas, \( p < 0.05 \), two-way ANOVA). CD11b immunoreactivity was virtually undetectable in uninjured mice (Fig. 2L).

Taken together, these findings show that the 2-cm drop produces abundant MAC deposition and inflammation in the brain. Therefore, the 2-cm height was chosen as standard weight drop in all subsequent experiments, to study the effect of MAC inhibition on the outcome of severe TBI.

C6 antisense inhibits liver C6 synthesis and reduces serum C6 levels

To define the role of MAC in TBI, we set out to block MAC formation in the severe closed head injury mouse model. To this end, we designed antisense oligonucleotides to target C6 production, thereby blocking MAC formation, but not early complement activation and C5a generation. We compared the injury-modulating effects of C6 antisense therapy with those of OmCl, which targets the terminal pathway at the level of C5, blocking both C5a and MAC production.

The antisense oligonucleotides used were modified with LNAs at the 5’ and 3’, and the central part consists of DNA to allow recruitment of RNase H (26). The whole oligo was phosphorothioate at the 5’ and 3’ ends, which limits the amount of phosphorothioates and associated toxicity. Trinitiation of the oligonucleotide allows monitoring of its biodistribution. The biodistribution of oligonucleotides is very predictable, with main uptake in the kidney and liver, and no uptake by the brain or peripheral nervous system (Fig. 3A). Because most C6 is synthesized in the liver (27), oligonucleotides are well suited for knockdown of C6 synthesis. In situ hybridization reveals that the oligonucleotide is distributed primarily in the Kupffer cells (Fig. 3B), which is normal for phosphorothioate oligonucleotides in rodents (28). Although the Kupffer cells showed the highest level of accumulation, lower levels of oligonucleotides accumulating in hepatocytes (28) are sufficient for knockdown of the C6 mRNA (synthesized by the hepatocytes) as shown in Fig. 3C and 3D. To test the optimal length of the oligonucleotides, we designed three short oligonucleotides against C6: an 11 mer, a 12 mer, and a 14 mer. These oligonucleotides were tested in BALB/c mice at increasing dosages from 0.5 up to 5 mg/kg. Both C6 mRNA levels in the liver (Fig. 3C) and C6 protein levels in serum (Fig. 3D) were monitored. The 14 mer oligonucleotide at doses of 3 and 5 mg/kg was efficient at knocking down C6 message in the liver and C6 levels in serum (\( p < 0.001 \), two-way ANOVA; Fig. 3D); this oligonucleotide at 5 mg/kg dose was therefore used in the following experiments. Because the mechanism of action of antisense oligonucleotides is based on the degradation of the targeted mRNA, it takes 3–5 d after treatment before the amount of circulating C6 protein is lowered. Furthermore, C6 levels remain low for many days after cessation of the treatment because the LNA antisense oligonucleotides are stable. Treatment (s.c.) of BALB/C mice with the 14 mer C6 antisense oligonucleotide for 4 consecutive days reduced C6 liver mRNA levels to 20% of controls by 5 d after end of treatment (\( p < 0.001 \), two-way ANOVA), remaining significantly lower than controls up to 35 d later (\( p < 0.01 \), two-way ANOVA; Fig. 3E). To check for sequence specificity of the antisense treatment, we monitored C3 levels and found them not to be affected, whereas a scrambled oligonucleotide did not inhibit C6 (Supplemental Fig. 2).

MAC inhibition reduces posttraumatic neurologic disability and weight loss

To test whether inhibition of MAC formation is protective in TBI, we monitored neurologic performance and weight loss (Fig. 4A, 4B) in mice treated with either C6-antisense or OmCl or PBS as control, up to 72 h after TBI.

Pretreatment of mice with the C6 antisense oligonucleotide (Supplemental Fig. 3A) significantly decreased liver C6 mRNA levels by \( \sim 85\% \) of PBS controls (\( p < 0.05 \), \( t \) test) (Supplemental Fig. 3B) and serum C6 protein levels by \( \sim 90\% \) of controls (\( p < 0.05 \), \( t \) test) at 6 d after end of treatment (Supplemental Fig. 3C).

Pretreatment with C6 antisense resulted in 50% reduction of NSS compared with PBS-treated controls at 1 h after TBI (\( p < 0.05 \), two-way ANOVA), reaching complete recovery at 72 h postinjury, a time when PBS-treated mice showed significant residual neurologic impairment (NSS 2.11 \( \pm \) 0.51; Fig. 4A). Weight loss was monitored in mice as an additional measure of clinical outcome. PBS-treated mice lost 18.7 \( \pm 1.9\% \) of their preinjury weight at 48 h postinjury, whereas pretreatment with C6 antisense significantly reduced weight loss to 7.8 \( \pm 1.7\% \) of uninjured levels (\( p < 0.05 \), two-way ANOVA), restoring pre-TBI weight at 72 h postinjury (Fig. 4A).

Mice treated with OmCl, administered immediately before the injury, showed complete neurologic disability (NSS score 10) at 1 h after TBI. However, they recovered significantly better than PBS-treated controls with NSS of 2.90 \( \pm 1.72 \) compared with 7.04 \( \pm 2.42 \) in controls (\( p < 0.05 \), two-way ANOVA) at 4 h postinjury; the improved recovery was maintained throughout the time course, reaching almost complete recovery at 72 h post-TBI (\( p < 0.05 \), two-way ANOVA). Treatment with OmCl administered 15 min after TBI also significantly improved neurologic outcome compared with PBS-treated controls at 4, 24, 48 and 72 h postinjury (\( p < 0.05 \), two-way ANOVA), whereas mice treated with OmCl at 30 min after TBI did not perform significantly better than controls at any of the time points examined (Fig. 4B).

Mice treated with OmCl immediately before TBI showed significantly lower weight loss at 48 h after TBI than PBS-treated mice (16.2 \( \pm 1.5\% \) PBS versus 14.5 \( \pm 1.5\% \) OmCl; \( p < 0.05 \), two-way ANOVA). Treatment with OmCl at 15 min after injury also significantly reduced weight loss at 48 h after TBI compared with PBS-treated mice (16.2 \( \pm 1.5\% \) PBS versus 10.4 \( \pm 0.9\% \) OmCl; \( p < 0.05 \), two-way ANOVA), whereas treatment at 30 min postinjury was not protective for weight loss (Fig. 4B).

Taken together, these findings show that pretreatment with C6 antisense or treatment with OmCl up to 15 min after TBI promote neurologic recovery and prevent weight loss after TBI.

MAC inhibition reduces inflammation at 72 h after TBI

To determine whether C6 antisense or OmCl treatment blocked MAC deposition and reduced inflammation in the brain, we performed immunohistochemistry for C9 (Fig. 5A–D) and Iba-1 (Fig. 5E–H) (see Table I) on sagittal sections of brains from inhibitor-treated-mice and controls. PBS treatment produced abundant and widespread deposition of MAC in the cerebellum, hippocampus, periventricular area, and subpial area of the brain as determined by immunostaining for C9 (Fig. 5A). Both C6 antisense and OmCl treatment reduced immunoreactivity for C9 in all brain areas examined (Fig. 5B, 5C). Quantification of the staining showed that C9 immunoreactivity covered 5.43 \( \pm 1.01 \) to 13.31 \( \pm 2.24\% \) of all examined brain areas in PBS-treated mice, whereas levels were significantly lower in mice treated with either C6
antisense (0.04 ± 0.02 to 0.33 ± 0.12%; p < 0.05, two-way ANOVA) or OmCI (0.38 ± 0.12 to 0.96 ± 0.44%; p < 0.05; Fig. 5D).

Immunostaining for Iba-1 in the PBS-treated brains showed abundant reactivity and enlarged microglia/macrophage morphology (Fig. 5E), whereas Iba-1 immunoreactivity was low in the brains of mice treated with either C6 antisense or OmCI (Fig. 5E, 5G). Quantification of the Iba-1 staining confirmed significantly lower levels of Iba-1 immunoreactivity in the cerebellum, periventricular, and subpial areas of brains from mice treated with C6 antisense (1.83 ± 0.79 to 2.13 ± 0.91%) or OmCI (1.30 ± 0.23 to 3.66 ± 0.80%) compared with PBS-treated controls (3.62 ± 0.39 to 6.98 ± 0.45%; p < 0.05, two-way ANOVA; Fig. 5H).

Taken together, these findings show that pretreatment with C6 antisense or treatment with OmCI up to 15 min after TBI block MAC deposition and reduce inflammation in the TBI brain at 72 h postinjury.

MAC inhibition reduces neuroaxonal loss at 72 h after TBI

Immunohistochemical examination using the cleaved caspase-3 marker of apoptosis (Fig. 6A–D) and the pan neurofilament marker of axons (see Table I) was performed in all brains (Fig. 6E–H).
to determine whether the significant improvement in neurologic performance observed after treatment with C6 antisense or OmCI is due to a reduction in neuroaxonal loss compared with PBS-treated controls. Neuroaxonal loss was most obvious in the cerebellum after severe TBI. Signs of neuronal apoptosis, shown by cleaved caspase-3 immunoreactivity, were evident in the granular layer of the cerebellum (Fig. 6B, arrows and inset), and evidence of axonal loss was detected in the Purkinje cells layer, in the granular layer, and in the white matter tracts of the cerebellum (Fig. 6F). Treatment with C6 antisense or OmCI up to 15 min after TBI preserved neurons and axons in the cerebellum as shown by minimal immunoreactivity for cleaved caspase-3 (Fig. 6C, inset, 6D, inset) and preserved axonal staining (Fig. 6G, 6H), which was comparable with that observed in uninjured controls (Fig. 6E).

Taken together, these findings show that pretreatment with C6 antisense or treatment with OmCI up to 15 min after TBI reduces apoptosis and prevents neuroaxonal loss in the TBI brain at 72 h postinjury.

Discussion

The complement system has been implicated in the neuroinflammatory and neurodegenerative sequelae, which follow the primary mechanical impact after TBI (3, 6–8, 10, 12). However, the specific role of the MAC in TBI has not previously been investigated. Our data clearly demonstrate that inhibition of MAC formation either by treatment with a C6 antisense oligonucleotide before TBI or by treatment with OmCI up to 15 min after TBI causes significant and substantial reduction of inflammation (up to 88% lower than placebo-treated controls) and neuroaxonal loss, especially in the cerebellum, resulting in improved neurologic performance (up to 92% better than placebo-treated controls) as measured by NSS.

TBI is a highly heterogeneous condition and, therefore, difficult to encompass in a single animal model. In this study, we used the closed head injury model, previously described by Flierl et al. (18), because it mimics the majority of clinically encountered injuries typically sustained on a closed skull during traffic accidents, violent attacks, or falls (29–31). In our setup, a weight drop of 2 cm produced a consistent and severe injury as determined by maximal NSS at 1 h after TBI. This is a more severe outcome than that reported by Flierl et al. for the same falling height, likely because of the smaller size of the mice used in this study, a factor known to affect the severity of head trauma (18). In addition, we demonstrated that the 2-cm drop on a closed skull results in substantial deposition of MAC and activation of microglia/macrophages throughout the brain within 72 h from the impact. By contrast, the weight drop of 1 cm produced only mild neurologic impairment, minimal MAC deposition, and hyperreactivity of microglia in the brain at 72 h posttrauma, supporting a recent report of extensive MAC deposition after severe focal penetrating injury, but not after mild diffuse rotational injury, in mice (6).

The complement system is a major component of the innate immune response and is the first line of defense against pathogens (32). Therefore, therapies aimed to inhibit complement activation should where possible target the detrimental effects of the complement cascade whereas maintaining its protective function to fight infections. This is especially critical for TBI patients who experience intensive care unit–acquired sepsis and respiratory...
failure far more frequently than other patients (33), and sepsis-associated mortality rate after injury is as high as 37% (34).

Therapeutic strategies to inhibit complement activation in TBI have targeted the complement cascade at its core functions, including generation of the opsonin C3b and potent anaphylatoxin C5a (7, 10–12), which are essential to fight infections. The MAC is the product of the terminal complement activation pathway. Thus, targeting MAC formation would maintain the function of the upstream complement components beneficial to mount an effective immune response against pathogens. Individuals unable to form the MAC, because of deficiencies of C6 or other terminal pathway proteins, are healthy, although they show increased susceptibility to Neisseria meningitidis infections (35), which must be prevented by antibiotic prophylaxis. Therefore, inhibition of the MAC is predicted to be a relatively safe therapeutic strategy in humans.

We have shown that MAC is a key determinant of axonal damage after crush injury of the peripheral nerve, exacerbating degeneration and impairing nerve regeneration and recovery of function (15). We also showed that rats deleted of the C6 component of the MAC are protected from early axonal loss and show improved nerve regeneration and recovery (17). Other groups have shown that MAC is a potent inducer of tissue injury also in the CNS, inducing upregulation of adhesion molecules and leukocyte infiltration (36), evoking epileptic seizures and neuronal cell death (37) in healthy rats, and contributing to demyelination and axonal injury in the EAE model of multiple sclerosis (24). Studies using mice deleted of the CD59a gene, the intrinsic regulator of MAC

**FIGURE 5.** MAC inhibition reduces inflammation at 72 h after TBI. Representative images of immunostainings for MAC with the anti-C9 Ab (A–C) and the pan microglia/macrophages with the anti–Iba-1 Ab (E–G) showing absent MAC deposition and reduced microglial reactivity in cerebellum of mice treated with terminal complement pathway inhibitors (PBS, n = 17; C6 antisense, n = 9; OmCI 15’ post-TBI, n = 13). Quantification of immunostainings for C9 (D) and Iba-1 (H) in cerebellum (cb), hippocampus (hc), periventricular area (pv), and subpial area (sp) of mice treated with complement inhibitors compared with PBS-treated control mice. Asterisks indicate statistically significant differences of C6 antisense-treated or OmCI-treated groups compared with PBS-treated controls; *p < 0.05.

**FIGURE 6.** MAC inhibition reduces apoptosis and axonal loss at 72 h after TBI. Representative images of immunostainings for apoptosis with the anticleaved caspase-3 Ab (A–D) and axons with the pan neurofilament Ab (E–H), showing apoptotic neurons in the granular layer (B) and loss of axons in the granular layer, white matter tract, and Purkinje cell layer (F) of the cerebellum from PBS-treated mice (n = 17). Apoptotic cells are occasionally detected in the brain of C6 antisense- (C, arrow in inset) (n = 9) or OmCI-treated mice (D, arrow in inset) (15 min after TBI, n = 13). No evident signs of axonal loss are detected in the C6 antisense- (G) or OmCI-treated mice (H). (E) Arrows indicate Purkinje cells, arrowheads indicate axons within the granular layer, and the asterisk indicates the white matter tract in the cerebellum from uninjured mice.
assembly, showed that CD59a-deficient mice are more susceptible to peripheral nerve trauma (16), ischemia/reperfusion injuries (38), EAE-induced demyelination and axonal injury (39), as well as neuropathology and neurologic impairment after closed head injury (8). Taken together, these studies support a detrimental role of MAC in the brain and point to a potential therapeutic effect of MAC inhibition.

The mechanisms of MAC generation and MAC-induced neurodegeneration after TBI are not fully clear. Early evidence, showing improved outcome after controlled cortical impact in mice treated with the C1 esterase inhibitor, point to the involvement of the classical and/or the lectin pathway (40), whereas the alternative pathway drives pathology (7, 12). It is possible that mechanical shearing forces, caused by TBI, expose axonal epitopes and/or flipped membranes that are recognized as “danger” signals by C1q, resulting in activation of the classical pathway and MAC formation via an Ab-independent manner (41). MAC then punches holes in the target membranes, releasing cell contents and driving further complement activation, including opsonization by the upstream complement components such as C3b/C4b. It is likely that iC3b then serves as a ligand for CR3-mediated clearance of damaged tissue (42, 43). We also observed MAC deposition on the surface of cells that are consistent with microglia/macrophages. It is known that sublytic amount of MAC can trigger cell activation and initiate signaling pathways that drive the expression of inflammatory molecules (44). We suggest that C9 immunoactivity on the surface of cells is indicative of deposition of sublytic MAC. Cell activation toward a proinflammatory profile would then contribute to tissue damage. In this study, we prove that inhibition of the MAC in mice, which have an otherwise functional complement system, is sufficient to inhibit microgli/macrophage activation, neuronal apoptosis, and axonal loss, improving neurologic performance. Notably, others have shown that sole inhibition of the C5a receptor (C5aR) significantly reduces pathology after TBI (11). The C5a-C5aR pathway has been shown to induce inflammation and tissue damage (45). Damaged tissue would, in turn, activate complement, amplifying the cascade and resulting in more MAC formation. Deposition of MAC in the tissue would exacerbate the damage. Therefore, blockade of the C5aR could also indirectly result in reduction of MAC formation, protecting from tissue damage. Furthermore, C5a functions also via the C5L2 receptor. Therefore, it is possible that blockade of the C5a/C5aR axis stimulates C5L2, which has been shown to drive anti-inflammatory pathways and reduce pathology in several disease models (46). The C5a-C5L2 axis may also have an effect on MAC formation and be protective in TBI.

To our knowledge, this is the first study to report the effects of therapeutic inhibition of MAC formation alone in neurotrauma, such as TBI. In addition, our findings demonstrate that systemic inhibition of C6 is sufficient to prevent local deposition of MAC in the brain. The LNA oligonucleotides, like all phosphorothioate-modified oligonucleotides, do not pass the BBB and primarily function by inhibition of C6 production in the liver. The resulting >80% knockdown of C6 mRNA in the liver caused a similar reduction in C6 protein production in the blood. Notably, the amount of loss of circulating C6 we achieve is sufficient for an effect in the CNS. Thus, these experiments show that it is possible to use drug targets, such as the terminal complement pathway, outside the nervous system for neurologic diseases. This is especially relevant for those conditions in which the BBB is initially intact.

Because C6 antisense therapy requires a few days to reduce C6 levels, this approach is not a treatment option for patients after the injury has occurred. Nevertheless, this study represents the proof of principle that sole inhibition of MAC formation is neuroprotective in TBI. Notably, reduced neuropathology and improved neurologic performance could be achieved by OmCI treatment administered up to 15 min after TBI, whereas a treatment delay of 30 min from the time of the injury showed no protection. These findings suggest that there is a therapeutic window of opportunity for timely intervention after TBI to prevent the secondary sequelae of neurologic damage by inhibiting the terminal complement pathway, raising the prospect that such strategies may work postinjury in humans. The length of the therapeutic window in humans is unknown, but comparison with studies in thrombolysis suggests it might be much longer than in rodents. As with thrombolitics, the “time is brain” concept likely applies, and treatment would be best given soon after insult by first responders at the site of the accident or in the ambulance (47). In summary, the data reported in this study support the hypothesis that specific inhibition of the MAC reduces neuronal apoptosis and axonal loss, and promotes recovery of neurologic performance after TBI. This is an attractive approach because of the specificity of the pathway being targeted, the observation that humans unable to form the MAC generally live a healthy life, and the prediction that a MAC-inhibiting drug could be prescribed as prophylactic treatment in patients at risk for injury or even after the injury has occurred, for example, caused by a traffic accident or a fall. Thus, our findings provide a rational basis for the development of a novel future therapeutic strategy for TBI.

Disclosures
V.R., K.F., and F.B. are shareholders (<5%) of Regenesance BV, a bio-pharmaceutical spin-off company that develops complement therapeutics. F.B. is Chief Scientific Officer of Regenesance BV.

References


