An anticomplement agent that homes to the damaged brain and promotes recovery after traumatic brain injury in mice

Marieta M. Ruseva[a,1,2], Valeria Ramaglia[b,1], B. Paul Morgan[a], and Claire L. Harris[a,3]

[a]Institute of Infection and Immunity, School of Medicine, Cardiff University, Cardiff CF14 4XN, United Kingdom; and [b]Department of Genome Analysis, Academic Medical Center, Amsterdam 1105 AZ, The Netherlands

Edited by Douglas T. Fearon, Cornell University, Cambridge, United Kingdom, and approved September 29, 2015 (received for review July 15, 2015)

Activation of complement is a key determinant of neuropathology and disability after traumatic brain injury (TBI), and inhibition is neuroprotective. However, systemic complement is essential to fight infections, a critical complication of TBI. We describe a targeted complement inhibitor, comprising complement receptor of the Ig superfamily (CRIg) fused with complement regulator CD59a, designed to inhibit membrane attack complex (MAC) assembly at sites of C3b/iC3b deposition. CRIg and CD59a were linked via the IgG2a hinge, yielding CD59-2a-CRIg dimer with increased iC3b/C3b binding avidity and MAC inhibitory activity. CD59-2a-CRIg inhibited MAC formation and prevented complement-mediated lysis in vitro. CD59-2a-CRIg dimer bound C3b-coated surfaces with submicromolar affinity (K_D). In experimental TBI, CD59-2a-CRIg administered post-trauma homed to sites of injury and significantly reduced MAC deposition, microglial accumulation, mitochondrial stress, and axonal damage and enhanced neurologic recovery compared with placebo controls. CD59-2a-CRIg inhibited MAC-induced inflammasome activation and IL-1β production in microglia. Given the important anti-infection roles of complement opsonization, site-targeted inhibition of MAC should be considered to promote recovery postneurotrauma.

traumatic brain injury | complement | therapy | CD59 | CRIg

Cognitive, behavioral, and emotional sequelae of traumatic brain injury (TBI) are largely due to neuroaxonal damage secondary to the mechanical injury (1, 2). Complement is an early determinant of posttraumatic neuroinflammation and secondary neuropathology (3–7); therefore, timely intervention to block complement activation may reduce neuroinflammation, neurodegeneration, and neurologic decline. Previous studies showed that complement inhibition protects in experimental TBI; however, most agents targeted the C3 convertase, the point of activation pathway convergence, resulting in broad inhibition of complement (8, 9). Others interfered with C5a, a potent inflammatory mediator (10), or with activation of the alternative pathway (11), a major player in the disease pathophysiology (12). Complement activation products are also key to effective immune defense against infections (13), a critical complication in TBI patients; therefore, the stage and location of complement inhibition is critically important for effective therapy in TBI, blocking detrimental effects while maintaining protective functions.

We recently demonstrated that inhibition of the most downstream complement activation product, the membrane attack complex (MAC), effectively prevents secondary neurologic damage and deficit in experimental TBI (14). MAC inhibition was achieved using an antisense oligonucleotide targeting C6, an essential MAC component, administered for 1 wk before injury (14). A clinically relevant anticomplement therapy in TBI must rapidly block complement activation when administered postinjury and be targeted to the activation site to minimize infection risk. We previously reported a murine CD59a-based targeted therapeutic using complement receptor of the Ig superfamily (CRIg), which binds C3 fragments, as the homing domain (15). In this article, we describe the development of this fusion protein, demonstrate its capacity to rapidly and specifically inhibit MAC at sites of complement activation, and test its therapeutic potential in experimental TBI. The construct, termed CD59-2a-CRIg, comprises CD59a linked to CRIg via the murine IgG2a hinge. CD59a prevents assembly of MAC in cell membranes (16), whereas CRIg binds C3b/iC3b deposited at sites of complement activation (17). The IgG2a hinge promotes dimerization to increase ligand avidity. CD59-2a-CRIg protected in the TBI model, demonstrating that site-targeted anti-MAC therapeutics may be effective in prevention of secondary neuropathology and improve neurologic recovery when administered post-TBI in patients.

Results

Generation and in Vitro Characterization of CD59-2a-CRIg. CD59-2a-CRIg was generated by expressing the extracellular domains of CD59a and CRIg linked through the murine IgG2a hinge to promote dimerization, thus increasing avidity for ligands (Fig. 1*A*). CD59-2a-CRIg, isolated from the culture supernatant of transfected CHO cells and analyzed by SDS/PAGE, migrated as two bands with an apparent molecular mass of 83 kDa and 45 kDa (Fig. 1*B*). These were separated by size exclusion chromatography (Superdex 200),

Significance

Traumatic brain injury (TBI) is the leading cause of death in young adults. We describe an inhibitor of complement membrane attack complex (MAC), which homes to sites of complement deposition in the injured brain, inhibits local inflammation and inflammasome activation, and is neuroprotective in experimental TBI, raising the prospect that such agents may provide effective treatment of TBI in man. Targeting anti-MAC therapy to pathology minimizes systemic effects and maintains upstream opsonization pathways essential to fight infections, a major cause of death post-TBI. Critically, we show a significant therapeutic window in the mouse model, raising the prospect that such a homing agent, administered postinjury, would be neuroprotective. We provide a rational basis for developing novel future therapeutic strategies for TBI.

Author contributions: B.P.M. and C.L.H. designed research; M.M.R. and V.R. performed research; M.M.R., V.R., and C.L.H. analyzed data; and M.M.R., V.R., B.P.M., and C.L.H. wrote the paper.

Conflict of interest statement: V.R. holds founding shares (<5%) in Regenesance BV, a biopharmaceutical company that develops complement inhibitors for clinical applications. C.L.H. is also employed by GlaxoSmithKline and has shares in GlaxoSmithKline; all work in her university lab was completed prior to that employment.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]M.M.R. and V.R. contributed equally to this work.

[2]Present address: Centre for Complement and Inflammation Research (CCIR), Division of Immunology and Inflammation, Department of Medicine, Imperial College London, London W12 0NN, United Kingdom.

[3]To whom correspondence should be addressed. Email: harriscl@cardiff.ac.uk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1513698112/-/DCSupplemental.

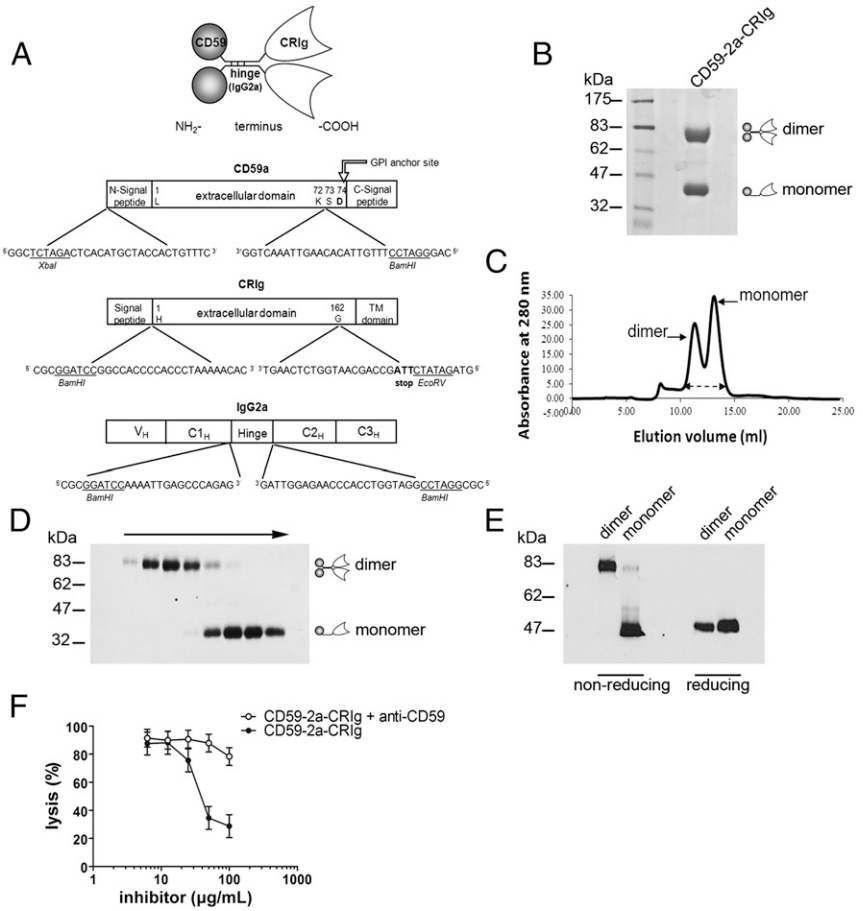

Fig. 1. Generation and functional analysis of CD59-2a-CRIg. (*A*) The extracellular domain of mouse CD59a was fused through the mouse IgG2a hinge to the N terminus of mouse CRIg to generate CD59-2a-CRIg. The sequences and positions of primers used for amplification are shown. The incorporated restriction sites are underlined, and the stop codon is in boldface. C_H, constant region of heavy chain; TM, transmembrane domain; V_H, variable region of heavy chain. (*B*) CD59-2a-CRIg was expressed in CHO cells, purified by anti-CD59a affinity chromatography, subjected to 7.5% nonreducing SDS/PAGE, and stained with Coomassie blue. Two major bands were observed, corresponding to monomeric and dimeric forms of CD59-2a-CRIg. (*C*) Purified protein was loaded onto a Superdex 200 column. CD59-2a-CRIg eluted as two peaks representing the dimer and monomer. (*D*) Eluted protein from the dimer and monomer peaks (indicated by the arrow) were analyzed by SDS/PAGE. Protein was visualized by silver staining. (*E*) Identity of the peaks was confirmed by Western blot analysis. Peak fractions were subjected to SDS/PAGE under nonreducing and reducing conditions, transferred to nitrocellulose, and then probed with rat anti-mouse CRIg mAb (MBI4, generated in house). (*F*) CD59-2a-CRIg complement inhibitory function was assessed in an alternative pathway hemolytic assay. The protective effect was abolished when CD59a moiety was blocked with anti-CD59 antibodies. Percent lysis was plotted against protein concentration.

yielding two peaks consistent with the expected masses of monomer and dimer (Fig. 1*C*). Both were recognized by anti-CRIg monoclonal antibody (mAb), and reducing SDS/PAGE demonstrated that the dimer was disulphide bonded (Fig. 1 *D* and *E*). The relative ratio of monomer:dimer, measured from the peak area, was 55%:45%.

The capacities of CD59-2a-CRIg to inhibit MAC formation and protect cells from complement lysis were assessed by haemolysis assay; guinea pig erythrocyte (GPE) and rat serum were used as the target and complement source, respectively. GPE was incubated with serial dilutions of CD59-2a-CRIg (55%:45%, dimer:monomer) and rat serum, dose titred to cause 90% lysis. CD59-2a-CRIg inhibited complement activation in a dose-dependent manner (Fig. 1*F*). Previous studies have reported that CRIg blocks alternative pathway activation in vitro (18). To confirm that haemolysis inhibition by CD59-2a-CRIg was due to CD59a inhibition of MAC formation, the CD59a moiety was blocked using anti-CD59a mAb; CD59a-blocked cells were no longer protected from lysis (Fig. 1*F*). To further test the function of each domain, CD59-2a-CRIg was incubated with GPE precoated with C3b and C5b67, and the free agent was washed away and the MAC developed with C3-deficient mouse serum. Lysis was blocked, demonstrating that CRIg localized the agent to the opsonized surface, and the CD59a domain blocked MAC formation (Fig. S1*A*).

We performed surface plasmon resonance (SPR) analysis to measure affinity of the CD59-2a-CRIg monomer (Fig. 2*A*) and dimer (Fig. 2*B*) for mouse C3b. A mouse C3b-opsonized chip surface was generated via the alternative pathway by flowing factor B and factor D over a surface coated with a nidus (50RU) of human C3b. C3bBb thus formed catalyzed cleavage of mouse C3 flowed across, resulting in abundant covalent deposition of mouse C3b (700RU). The equilibrium dissociation constant (K_D) for the monomer and dimer was determined by steady-state analysis. The apparent affinity (K_D) was 4.65 μM for monomer and eightfold higher (0.55 μM) for the dimer, demonstrating the impact of dimerization on affinity of CD59-2a-CRIg for the C3b ligand and confirming that both CRIg domains bind simultaneously to separate C3b molecules to deliver avidity.

In Vivo Localization of CD59-2a-CRIg. To test CD59-2a-CRIg homing to the site of injury, anti-CRIg mAb was used to localize the agent in brains of treated mice at 72 h post-TBI (Fig. 3). Mice subjected to TBI were treated with 10 or 20 mg/kg CD59-2a-CRIg (mixture of dimer and monomer), or PBS, immediately postinjury (i.v.), according to the described protocol (*Materials and Methods*). Analysis of consecutive brain sections showed localization of CD59-2a-CRIg to the impact site in mice treated with 20 mg/kg CD59-2a-CRIg (Fig. 3*A*); localization was not detected at 10 mg/kg, indicating that the higher dose was required to adequately target the injury site. The C3 activation fragment (C3b/iC3b/C3dg) deposition colocalized with CD59-2a-CRIg (Fig. 3*B*), but immunostaining for MAC (14, 19–22) was negative in corresponding areas (Fig. 3*C*); in contrast, brain tissue from PBS-treated TBI controls showed C3 fragments (Fig. 3*E*) and MAC (Fig. 3*F*) deposited at the site of injury, demonstrating blockade of MAC formation by CD59-2a-CRIg in situ. Anti-CRIg mAb did not detect endogenous CRIg in mouse brain (Fig. 3*D*), likely because of low expression in brain parenchyma (23). Together, these data demonstrate that CD59-2a-CRIg localizes to areas of complement activation and inhibits MAC deposition in vivo.

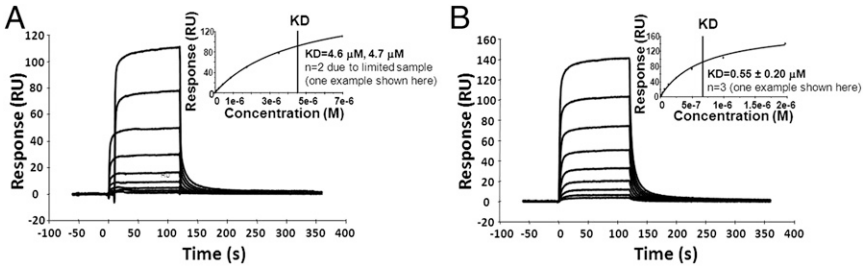

Fig. 2. Dimerization of CD59-2a-CRIg increased the affinity for C3b. CD59-2a-CRIg monomer (*A*) and dimer (*B*) were flowed over a C3b-coated chip at concentrations between 30 nM and 7.0 μM (monomer) in duplicate and 8 nM–1.98 μM (dimer) in triplicate (K_D is mean ± SD). The equilibrium binding response was measured. Apparent affinity (K_D) was determined by steady-state analysis (*Insets* show one example).

CD59-2a-CRIg Reduces Posttraumatic Neurologic Disability and Weight Loss. To test whether MAC inhibition by CD59-2a-CRIg is protective in TBI, neurological performance and weight loss were monitored up to 72 h postinjury in mice treated with CD59-2a-CRIg or PBS as the control; sham-operated controls were also included (Fig. S2). Anti-C5 therapies that blocked both C5a and MAC were previously shown to be effective when delivered up to 15 min postinjury (14). To test whether this therapeutic window could be expanded, mice were treated with CD59-2a-CRIg 30 min post-TBI and with a second dose 24 h later. Treatment resulted in 50% reduction of the neurological severity score (NSS) at 4 h compared with PBS controls ($P < 0.001$, two-way ANOVA); improvement was maintained throughout the time course and nearly complete at 72 h postinjury, when controls remained neurologically impaired (NSS, 2.8 ± 0.7). Sham-operated controls showed no neurological impairment (Fig. 4*A*). Weight loss was monitored as an additional clinical outcome. PBS-treated mice lost 13.3 ± 3.6% of preinjury weight at 48 h postinjury, whereas mice treated with CD59-2a-CRIg displayed significantly reduced weight loss (6.4 ± 3.7%; $P < 0.01$, two-way ANOVA), regaining pre-TBI weight by 72 h. Sham-operated controls did not lose weight (Fig. 4*B*). Together, these findings show that treatment with CD59-2a-CRIg administered from 30 min post-TBI promotes recovery after TBI.

CD59-2a-CRIg Reduces Inflammation, Mitochondrial Stress, and Axonal Injury Post-TBI. To determine whether CD59-2a-CRIg blocked MAC deposition and reduced brain inflammation, sagittal sections from treated and control mice were stained for C9, a marker of MAC deposition (14, 19–22) (Fig. 5 *A–C*), and Iba-1, a marker of microglia/macrophages (14) (Fig. 5 *D–F*). Brain sections in sham controls were negative for MAC staining (Fig. 5*A* and Figs. S3*A* and S4*A*), whereas PBS-treated TBI mice displayed abundant, widespread MAC deposition in the cortex (Fig. 5*B*), hippocampus (Fig. S3*B*), and periventricular areas (Fig. S4*B*). Treatment with CD59-2a-CRIg reduced MAC immunoreactivity in all brain areas examined (Fig. 5*C* and Figs. S3*C* and S4*C*). Iba-1 immunostaining in sham controls showed few, small immunolabeled cells (Fig. 5*D* and Figs. S3*D* and S4*D*). PBS-treated TBI brains showed abundant, large Iba-1–positive cells resembling reactive microglia (Fig. 5*E* and Figs. S3*E* and S4*E*); Iba-1 immunoreactivity was low in brains of TBI mice treated with CD59-2a-CRIg (Fig. 5*F* and Figs. S3*F* and S4*F*), demonstrating reduced inflammation.

To determine whether the observed improvement in neurological performance post-TBI in mice treated with CD59-2a-CRIg is due to reduced neuronal damage, immunohistochemistry using mitochondrial heat shock protein 70 (mtHSP70; mitochondrial stress marker) (Fig. 5 *G–J*) and amyloid precursor protein (APP; axonal injury marker) (Fig. 5 *J–L*) was performed in all brains. Sham controls showed no signs of mitochondrial stress (Fig. 5*G* and Figs. S3*G* and S4*G*) or axonal injury (Fig. 5*J* and Figs. S3*J* and S4*J*). Staining for mtHSP70 and APP was strongest in PBS-treated TBI mice, in neurons located in proximity to the site of impact (Fig. 5 *H* and *K*), in the hippocampal CA1 region (Fig. S3 *H* and *K*), and in the perivascular area (Fig. S4 *H* and *K*). In contrast, mtHSP70 and APP immunoreactivities were low in brains of CD59-2a-CRIg–treated TBI mice (Fig. 5 *I* and *L* and Figs. S3 *I* and *L* and S4 *I* and *L*), demonstrating that treatment reduced mitochondrial stress and axonal injury. Quantification of MAC staining showed abundant immunoreactivity (9.83 ± 1.07%; range, 2.15–17.50%) of all examined brain areas in PBS-treated TBI mice; staining was significantly lower in CD59-2a-CRIg–treated TBI mice (0.51 ± 0.12%; range, 0.23–2.70%; $P < 0.001$, two-way ANOVA) (Fig. 5*M*). Iba-1 immunoreactivity covered 4.97 ± 0.18% (range, 2.92–6.35%) of the brain areas in PBS-treated TBI mice but was significantly lower in all areas in CD59-2a-CRIg–treated TBI mice (1.12 ± 0.03%; range, 0.78–1.31%; $P <$ 0.01–0.001, two-way ANOVA) (Fig. 5*N*). Quantification of immunostaining for mtHSP70 and APP showed that CD59-2a-CRIg treatment significantly reduced mitochondrial stress and axonal

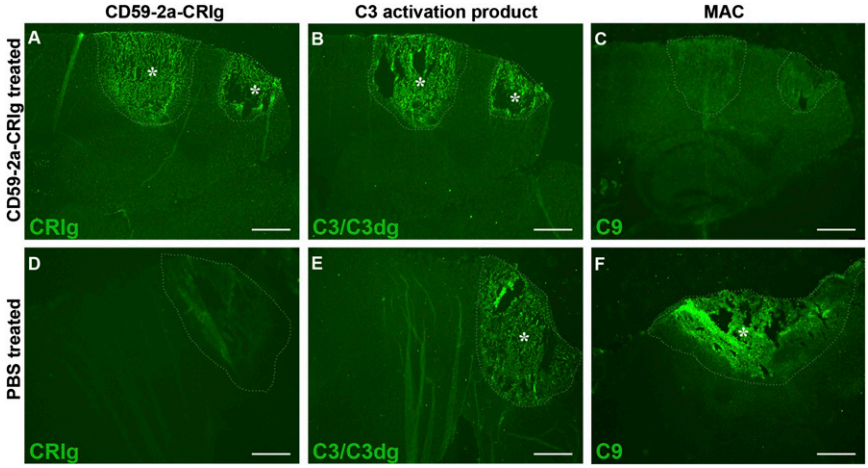

Fig. 3. CD59-2a-CRIg homed to opsonized tissue and inhibited MAC deposition in vivo. Mice were treated immediately after TBI with either CD59-2a-CRIg (20 mg/kg, i.v., $n = 2$) (*A–C*) or PBS ($n = 3$) as the control (*D–F*). Brains were collected at 3 d postinjury and consecutive sections immunostained for CRIg to localize the CD59-2a-CRIg in situ (*A* and *D*), for C3/C3dg to detect opsonized tissue (*B* and *E*), and for C9 to detect MAC deposition (*C* and *F*). CD59-2a-CRIg localized to areas of tissue injury at the impact sites (asterisk in *A*) and was absent in PBS-treated mice (*D*). C3 activation products (C3b/iC3b/C3dg) present at impact sites (asterisks in *B* and *E*) colocalized with CD59-2a-CRIg in treated mice (*B*). MAC deposition was absent in the CD59-2a-CRIg–treated group (*C*) but abundant at the impact site in controls (asterisk in *F*). In all images, the impact site is enclosed in the dotted line. (Scale bar, 500 μm.)

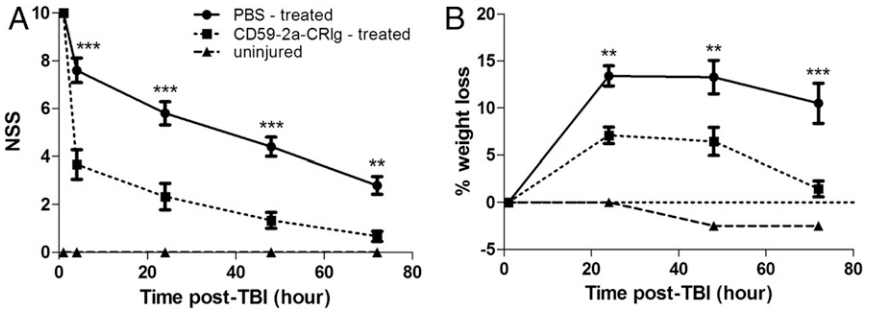

Fig. 4. CD59-2a-CRIg reduced neurologic disability and weight loss after TBI. Shown are the NSS (*A*) and weight loss (*B*) of mice treated with either CD59-2a-CRIg (20 mg/kg) ($n = 6$) or PBS ($n = 5$) 30 min after TBI and 24 h later. Asterisks indicate statistically significant differences, with $^{**}P < 0.01$ or $^{***}P < 0.001$ determined by two-way ANOVA with Bonferroni post hoc test.

injury in the cortex (mtHSP70, 3.25 ± 0.08% vs. 0.34 ± 0.03%, $P < 0.001$; APP, 3.76 ± 0.06% vs. 1.74 ± 0.07%, $P < 0.001$, two-way ANOVA), hippocampus (mtHSP70, 2.55 ± 0.20% vs. 0.05 ± 0.01%, $P < 0.001$; APP, 2.02 ± 0.11% vs. 0.44 ± 0.08%, $P < 0.001$, two-way ANOVA), and periventricular area (mtHSP70, 1.37 ± 0.12% vs. 0.12 ± 0.02%, $P < 0.001$; APP, 2.92 ± 0.12% vs. 0.41 ± 0.07%, $P < 0.001$, two-way ANOVA) (Fig. 5 *O* and *P*). These findings show that CD59-2a-CRIg, administered 30 min post-TBI and 24 h later, homes to sites of tissue injury, blocks MAC deposition, and reduces inflammation, neuronal stress, and further injury at 72 h post-TBI.

CD59-2a-CRIg Inhibits Inflammasome Activation Post-TBI. Precisely how MAC induces nerve damage after TBI is unknown. Recent reports showed that sublethal MAC triggers NLRP3 (nucleotide-binding domain, leucine-rich repeat containing family, pyrin domain-containing 3) inflammasome activation and release of IL-1β (24, 25). We therefore tested whether MAC regulates secondary inflammatory processes after TBI by inducing inflammasome activation in brain cells, leading to production of IL-1β, which is known to be toxic to neurons (26, 27). In situ hybridization demonstrated expression of *IL-1β* mRNA in injured brains of controls at 72 h post-TBI. The *IL-1β* signal localized on Iba-1–positive

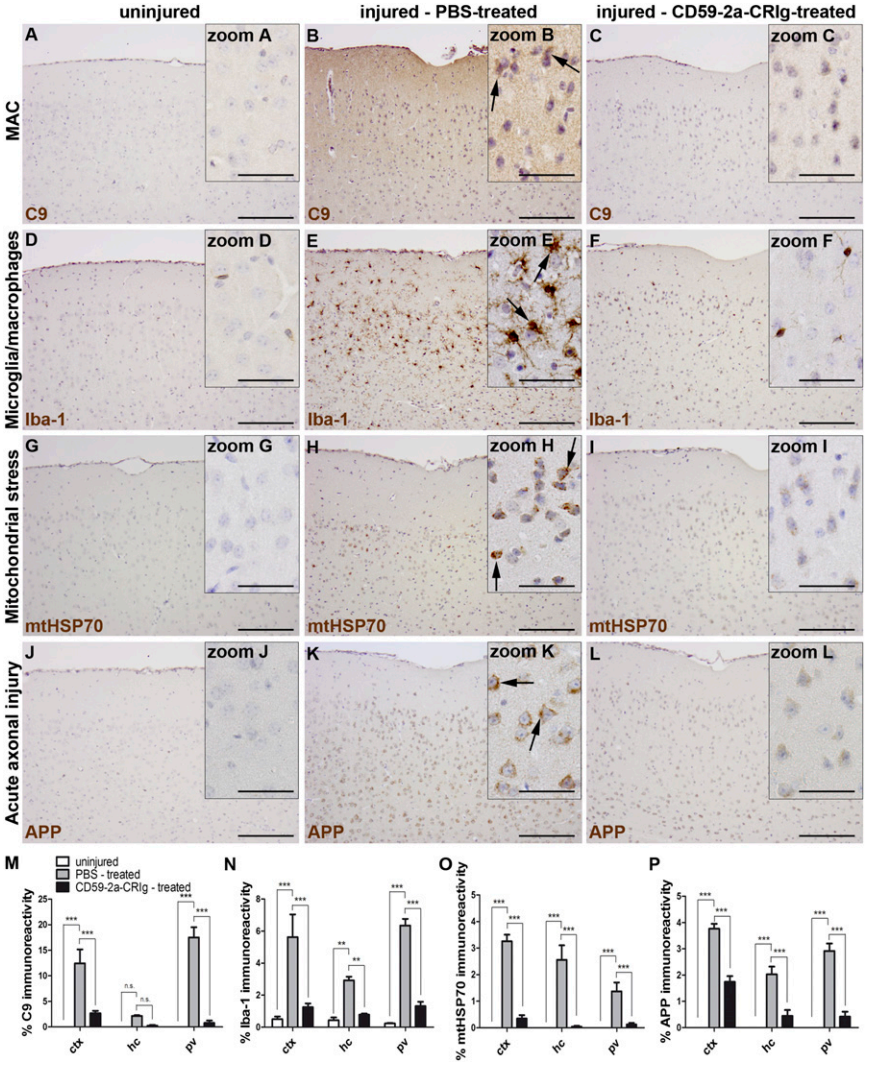

Fig. 5. CD59-2a-CRIg reduced MAC deposition, inflammation, and neuronal damage in the cortex at 3 d post-TBI. (*A–L*) Representative images of immunostaining for C9/MAC (*A–C*), Iba-1 marker of microglia/macrophages (*D–F*), mtHSP70 marker of mitochondrial stress (*G–I*), and the APP marker of acute axonal injury (*J–L*) in the cortex of uninjured mice (*A*, *D*, *G*, and *J*; $n = 3$) or injured mice treated with either PBS (*B*, *E*, *H*, and *K*; $n = 5$) or CD59-2a-CRIg (*C*, *F*, *I*, and *L*; 20 mg/kg i.v.; $n = 6$) at 30 min post-TBI and 24 h later, then killed at 72 h. Note in the PBS-treated group intense C9/MAC staining in close proximity to neurons (arrows in *zoom B*), Iba-1 immunoreactive cells closely apposed to neurons (arrows in *zoom E*), and mtHSP70 and APP immunoreactivity in neuronal cell bodies (arrows in *zoom H* and *zoom K*). [Scale bar, (*A–L*) 200 μm and (*Insets*) 50 μm.] (*M–P*) Quantification of C9/MAC (*M*), Iba-1 (*N*), mtHSP70 (*O*), and APP (*P*) immunoreactivity in mouse brains showing that the intensity of staining is significantly reduced for each marker in the cortex (ctx), hippocampus (hc), and periventricular area (pv) of CD59-2a-CRIg–treated mice compared with PBS-treated controls. Statistically significant differences with $^{**}P < 0.01$ or $^{***}P < 0.001$ are determined by two-way ANOVA with Bonferroni post hoc test.

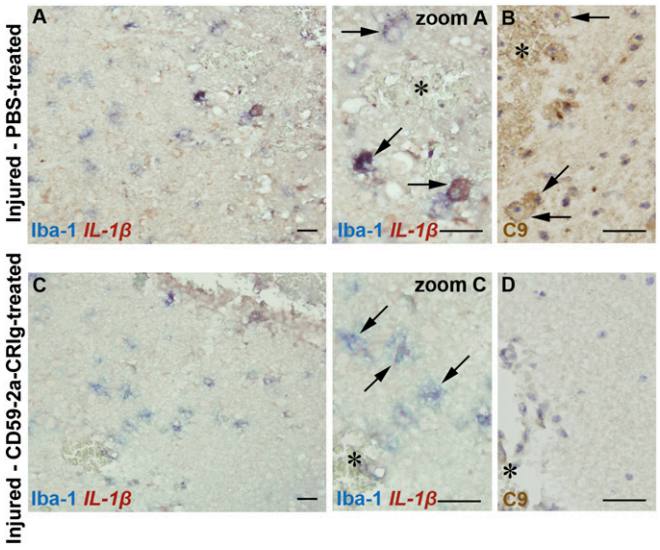

Fig. 6. CD59-2a-CRIg reduced IL-1β production by microglia/macrophages at 3 d post-TBI. Shown are representative images of immunostaining for the Iba-1 marker of microglia/macrophages, in situ hybridization for *IL-1β* mRNA, and immunostaining for C9/MAC in the brains of injured mice treated with either PBS (*A* and *B*; $n = 5$) or CD59-2a-CRIg (*C* and *D*; 20 mg/kg i.v.; $n = 6$) at 30 min post-TBI and 24 h later. In PBS-treated mice, the Iba-1–positive microglia/macrophages (*A*, blue) colocalized with the *IL-1β* mRNA signal (*A*, red) especially in areas of edema, indicated by the extravasation of erythrocytes (asterisk in *zoom A*). Cells coated with MAC (arrows in *B*, red) were also found in consecutive sections, in close proximity to edema (asterisk in *B*). In the CD59-2a-CRIg–treated mice, Iba-1–positive microglia/macrophages (*C*, blue) were negative for *IL-1β* mRNA in equivalent brain areas in close proximity to edema (asterisk in *zoom C*). No MAC signal (*D*) was detected in consecutive sections, in close proximity to edema (asterisk in *D*). In *B* and *D*, nuclei are stained with hematoxylin (in blue). (Scale bar in *A*–*D* and zoom, 25 μm.)

cells in brain areas located in close proximity to edema (Fig. 6 *A*, *zoom A*). These areas were also enriched in MAC-coated cells (Fig. 6*B*), indicating that MAC triggers inflammasome activation and IL-1β production in microglia. Treatment with CD59-2a-CRIg post-TBI eliminated the *IL-1β* signal (Fig. 6*C*) and MAC staining (Fig. 6*D*) in equivalent brain areas containing Iba-1–positive cells. These findings show that CD59-2a-CRIg administered after TBI inhibits MAC-induced inflammasome activation and IL-1β production in microglia in the TBI brain.

Discussion

Clinical and experimental studies have implicated complement in the neuroinflammatory and neurodegenerative sequelae to primary mechanical injury after TBI (3, 6, 7, 9, 11, 28). Therapeutic strategies involving systemic complement inhibition have proven successful in experimental TBI (6, 9–11) but have limited clinical relevance because of infection risk. Here we describe an anticomplement therapeutic, CD59-2a-CRIg, that homed to sites of brain injury in TBI, blocked MAC formation, and delivered neuroprotection. This strategy improves bioavailability; minimizes adverse effects due to systemic inhibition; specifically targets the MAC, thereby maintaining upstream activities; and provides membrane localization, which is necessary for optimal CD59a function (29).

Complement is a major component of innate immunity and the first-line defense against pathogens (13). Anticomplement therapies should, ideally, target detrimental effects of complement while maintaining anti-infection function, especially critical for TBI patients who suffer hospital-acquired sepsis far more frequently than other patients (30), with an associated mortality rate as high as 37% (31). Notably, selective inhibition of MAC in rodent models of brain injury reduced pathology and clinical disability (20, 22). In TBI, the pathological role of the MAC had not been tested due to lack of specific MAC inhibitors; however, the recent description of a C6 antisense enabled testing (14). When administered before TBI, C6 antisense significantly reduced inflammation and neuroaxonal loss and improved the neurologic outcome. C6 antisense therapy requires several days pretreatment to reduce C6 levels, so it is not a treatment option for TBI patients where a drug must be effective quickly when administered postinjury; an ideal drug will also spare upstream complement functions and home to the site of injury, thereby minimizing systemic effects.

Homing agents that use a fragment of complement receptor 2 (CR2) for delivery to complement-opsonized tissue sites have been described (32, 33). CR2 binds iC3b and, preferentially, C3dg; fusion proteins comprising the C3dg-binding fragment of CR2 linked to a complement regulator delivered therapy with reduced infection risk when administered systemically in models (33). We generated CD59a-based targeted therapeutics using CRIg as the targeting domain and the IgG2a hinge to promote dimerization (15). Preliminary analysis demonstrated that CD59a positioned N-terminally relative to CRIg had better MAC inhibitory function compared with constructs with C-terminal CD59a (Fig. S1*B*). CRIg binds iC3b at a site within the C3c fragment; we propose that this alternative targeting domain will preferentially distribute the homing agent to tissues where iC3b predominates over C3dg. Thus, a CRIg-targeted agent may home to sites of ongoing, or recent, complement activation and not to sites of previous complement activation where C3dg may persist (34). An additional advantage, conferred by the IgG2a hinge, is avidity; dimerization will improve bioavailability and homing efficacy. This effect was confirmed by SPR-dimeric CD59-2a-CRIg bound target ($K_D = 0.55$ μM) with an eightfold higher affinity than monomeric CD59-2a-CRIg. Kinetic analysis of binding of another CRIg-targeted reagent, CRIg-FH, to C3b was previously published (17); however, a direct comparison with CD59-2a-CRIg cannot be made because both CRIg and FH moieties of CRIg-FH bind C3b and contribute to the interaction.

We previously described a mouse closed skull TBI model; a 2-cm weight drop caused severe and widespread inflammation and neuropathology not only at the impact site but also in deeper areas including the hippocampus and paraventricular area (14). Post-TBI treatment with CD59-2a-CRIg inhibited inflammation and neuroaxonal damage throughout the brain, showing that this targeted anti-MAC therapeutic inhibited the secondary wave of pathology that follows primary mechanical injury. Critically, we show that CD59-2a-CRIg colocalized with its ligands (C3b/iC3b) in the injured brain, reduced neuropathology, and improved neurologic performance when administered 30 min after TBI. Whether administration of CD59-2a-CRIg may be effective when administered even later postinjury is an important question requiring further exploration. The data suggest a therapeutic window of opportunity for effective intervention after TBI to prevent secondary neurological damage, raising the prospect that such strategies may work when administered immediately postinjury in humans, perhaps by first responders at the scene or in the ambulance, as currently happens with thrombolytics with a reported 4.5-h window (35).

The mechanisms of complement activation and MAC-induced neurodegeneration after TBI are uncertain. Early reports showing improved outcomes after TBI in mice treated with C1 inhibitor implicate the classical and/or lectin pathway (36), whereas others implicate the alternative pathway (6, 11). Cell damage caused by mechanical shearing in TBI may expose axonal epitopes, cell contents, and/or flipped membranes, all "danger" signals recognized by C1q, resulting in antibody-independent classical pathway activation and MAC formation (37). MAC then breaches target membranes, releasing cell contents and driving further complement activation and opsonization of targets by C3b/iC3b, providing ligands for CR3-mediated phagocytic clearance of damaged tissue (38, 39). We observed MAC deposition on microglia in TBI, especially in

proximity to a leaky blood brain barrier. Recent studies have shown that MAC can elicit secretion of proinflamamatory cytokines such as IL-1β and IL-18 in vitro and in vivo via NLRP3 inflammasome activation (24, 25). Here we show that MAC-positive microglia express mRNA for IL1-β, implicating sublytic MAC in driving cells toward a proinflammatory state that may contribute to tissue damage, endothelial permeability, and expression of adhesion molecules (40), facilitating recruitment of inflammatory cells into the brain.

In summary, we show that CD59-2a-CRIg homes to opsonized surfaces and protects from MAC-mediated damage in vitro and in vivo in the TBI model, the latter with a therapeutic window that permits treatment postinjury. A homing MAC inhibitor is attractive for therapy because it minimizes systemic effects and risk of infection. Our findings provide a rational basis for a novel future therapeutic strategy for TBI.

Materials and Methods

Preparation of CD59-2a-CRIg. DNA encoding the signal peptide and the extracellular domain of mouse CD59a (C-terminal residue Lys72) was cloned into expression vector pDR2ΔEF1α (a gift from I. Anegon, INSERM U437, Nantes, France) (41). DNA encoding the extracellular domain of mouse CRIg (residues 1–162) was cloned downstream of CD59. To generate CD59-2a-CRIg, DNA encoding the hinge region of mouse IgG2a was cloned between CD59Aand CRIg. CD59-2a-CRIg was isolated from the supernatant of stably transfected CHO cell clones cultured in CELLine AD1000 (Integra Biosciences) using anti-CD59 (clone 7A6) affinity column. Purified protein was analyzed by 7.5% SDS/PAGE.

Closed Head Injury Model. Focal TBI was induced as described using a standardized free-falling weight drop device (42).

Animals. All experiments were approved by the Academic Medical Center Animal Ethics Committee (DNL 102705) and complied with Dutch national guidelines for the care of experimental animals.

Statistical Analysis. All statistical analyses were performed using GraphPad Prism 7.0 analysis software. Two-way ANOVA, including Bonferroni correction and post hoc tests, was used for all tests with statistically significant differences when $P < 0.05$. All values are given as means ± SEM unless otherwise indicated.

ACKNOWLEDGMENTS. We thank Iliana Michailidou for technical support with in situ hybridization. This work was funded by a Cardiff University PhD studentship (to M.M.R.) and Brain Foundation of the Netherlands Fellowship F2010 (1)-05 (to V.R.).

1. Max JE, Robertson BA, Lansing AE (2001) The phenomenology of personality change due to traumatic brain injury in children and adolescents. *J Neuropsychiatry Clin Neurosci* 13(2):161–170.
2. Salmond CH, Sahakian BJ (2005) Cognitive outcome in traumatic brain injury survivors. *Curr Opin Crit Care* 11(2):111–116.
3. Bellander BM, Singhrao SK, Ohlsson M, Mattsson P, Svensson M (2001) Complement activation in the human brain after traumatic head injury. *J Neurotrauma* 18(12):1295–1311.
4. Burk AM, et al. (2012) Early complementopathy after multiple injuries in humans. *Shock* 37(4):348–354.
5. Stahel PF, et al. (2001) Intrathecal levels of complement-derived soluble membrane attack complex (sC5b-9) correlate with blood-brain barrier dysfunction in patients with traumatic brain injury. *J Neurotrauma* 18(8):773–781.
6. Leinhase I, et al. (2006) Reduced neuronal cell death after experimental brain injury in mice lacking a functional alternative pathway of complement activation. *BMC Neurosci* 7:55.
7. Stahel PF, et al. (2009) Absence of the complement regulatory molecule CD59a leads to exacerbated neuropathology after traumatic brain injury in mice. *J Neuroinflammation* 6:2.
8. Kaczorowski SL, Schiding JK, Toth CA, Kochanek PM (1995) Effect of soluble complement receptor-1 on neutrophil accumulation after traumatic brain injury in rats. *J Cereb Blood Flow Metab* 15(5):860–864.
9. Leinhase I, et al. (2006) Pharmacological complement inhibition at the C3 convertase level promotes neuronal survival, neuroprotective intracerebral gene expression, and neurological outcome after traumatic brain injury. *Exp Neurol* 199(2):454–464.
10. Sewell DL, et al. (2004) Complement C3 and C5 play critical roles in traumatic brain cryoinjury: Blocking effects on neutrophil extravasation by C5a receptor antagonist. *J Neuroimmunol* 155(1-2):55–63.
11. Leinhase I, et al. (2007) Inhibition of the alternative complement activation pathway in traumatic brain injury by a monoclonal anti-factor B antibody: A randomized placebo-controlled study in mice. *J Neuroinflammation* 4:13.
12. Holers VM (2008) The spectrum of complement alternative pathway-mediated diseases. *Immunol Rev* 223:300–316.
13. Ricklin D, Hajishengallis G, Yang K, Lambris JD (2010) Complement: A key system for immune surveillance and homeostasis. *Nat Immunol* 11(9):785–797.
14. Fluiter K, Opperhuizen AL, Morgan BP, Baas F, Ramaglia V (2014) Inhibition of the membrane attack complex of the complement system reduces secondary neuroaxonal loss and promotes neurologic recovery after traumatic brain injury in mice. *J Immunol* 192(5):2339–2348.
15. Ruseva MM, Morgan BP, Harris CL (2009) Targeting sites of complement activation by engineering selective drug delivery. *Mol Immunology* 46:2865.
16. Meri S, et al. (1990) Human protectin (CD59), an 18,000-20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. *Immunology* 71(1):1–9.
17. Qiao Q, et al. (2014) A novel CRIg-targeted complement inhibitor protects cells from complement damage. *FASEB J* 28(11):4986–4999.
18. Helmy KY, et al. (2006) CRIg: A macrophage complement receptor required for phagocytosis of circulating pathogens. *Cell* 124(5):915–927.
19. Pratt JR, Basheer SA, Sacks SH (2002) Local synthesis of complement component C3 regulates acute renal transplant rejection. *Nat Med* 8(6):582–587.
20. Mead RJ, Singhrao SK, Neal JW, Lassmann H, Morgan BP (2002) The membrane attack complex of complement causes severe demyelination associated with acute axonal injury. *J Immunol* 168(1):458–465.
21. Piddlesden SJ, et al. (1994) Soluble recombinant complement receptor 1 inhibits inflammation and demyelination in antibody-mediated demyelinating experimental allergic encephalomyelitis. *J Immunol* 152(11):5477–5484.
22. Ramaglia V, et al. (2007) The membrane attack complex of the complement system is essential for rapid Wallerian degeneration. *J Neurosci* 27(29):7663–7672.
23. Ma Y, et al. (2015) Protein kinase cα regulates the expression of complement receptor Ig in human monocyte-derived macrophages. *J Immunol* 194(6):2855–2861.
24. Laudisi F, et al. (2013) Cutting edge: The NLRP3 inflammasome links complement-mediated inflammation and IL-1β release. *J Immunol* 191(3):1006–1010.
25. Triantafilou K, Hughes TR, Triantafilou M, Morgan BP (2013) The complement membrane attack complex triggers intracellular Ca2+ fluxes leading to NLRP3 inflammasome activation. *J Cell Sci* 126(Pt 13):2903–2913.
26. Rossi S, et al. (2014) Interleukin-1β causes excitotoxic neurodegeneration and multiple sclerosis disease progression by activating the apoptotic protein p53. *Mol Neurodegener* 9:56.
27. Lai AY, Swayze RD, El-Husseini A, Song C (2006) Interleukin-1 beta modulates AMPA receptor expression and phosphorylation in hippocampal neurons. *J Neuroimmunol* 175(1-2):97–106.
28. Rostami E, et al. (2013) The terminal pathway of the complement system is activated in focal penetrating but not in mild diffuse traumatic brain injury. *J Neurotrauma* 30(23):1954–1965.
29. Zhang HF, Yu J, Bajwa E, Morrison SL, Tomlinson S (1999) Targeting of functional antibody-CD59 fusion proteins to a cell surface. *J Clin Invest* 103(1):55–61.
30. Mascia L, et al.; Sepsis Occurrence in Acutely Ill Patients (SOAP) Investigators (2008) Extracranial complications in patients with acute brain injury: A post-hoc analysis of the SOAP study. *Intensive Care Med* 34(4):720–727.
31. Plurad DS, et al. (2010) The association of race and survival from sepsis after injury. *Am Surg* 76(1):43–47.
32. Song H, et al. (2003) Complement receptor 2-mediated targeting of complement inhibitors to sites of complement activation. *J Clin Invest* 111(12):1875–1885.
33. Atkinson C, et al. (2005) Targeted complement inhibition by C3d recognition ameliorates tissue injury without apparent increase in susceptibility to infection. *J Clin Invest* 115(9):2444–2453.
34. Zhang R, Zheng ZY, Lin JS, Qu LJ, Zheng F (2012) The continual presence of C3d but not IgG glomerular capillary deposition in stage I idiopathic membranous nephropathy in patients receiving corticosteroid treatment. *Diagn Pathol* 7:109.
35. Dorado L, Millán M, Dávalos A (2014) Reperfusion therapies for acute ischemic stroke: An update. *Curr Cardiol Rev* 10(4):327–335.
36. Longhi L, et al. (2009) C1-inhibitor attenuates neurobehavioral deficits and reduces contusion volume after controlled cortical impact brain injury in mice. *Crit Care Med* 37(2):659–665.
37. Baas F, Ramaglia V (2014) Uncovering the path to neurodegeneration from playingfield to battlefield. *Traumatic Brain Injury*, eds Sadaka F, Quinn T (Intech, Rijeka, Croatia), pp 79–106.
38. Reichert F, Rotshenker S (2003) Complement-receptor-3 and scavenger-receptor-AI/II mediated myelin phagocytosis in microglia and macrophages. *Neurobiol Dis* 12(1):65–72.
39. Rotshenker S (2003) Microglia and macrophage activation and the regulation of complement-receptor-3 (CR3/MAC-1)-mediated myelin phagocytosis in injury and disease. *J Mol Neurosci* 21(1):65–72.
40. Rothwell NJ, Luheshi GN (2000) Interleukin 1 in the brain: Biology, pathology and therapeutic target. *Trends Neurosci* 23(12):618–625.
41. Charreau B, et al. (1994) Protection of rat endothelial cells from primate complement-mediated lysis by expression of human CD59 and/or decay-accelerating factor. *Transplantation* 58(11):1222–1229.
42. Flierl MA, et al. (2009) Mouse closed head injury model induced by a weight-drop device. *Nat Protoc* 4(9):1328–1337.
43. Morgan BP (2000) Measurement of complement hemolytic activity, generation of complement-depleted sera, and production of hemolytic intermediates. *Methods Mol Biol* 150:61–71.
44. Harris CL, et al. (2003) Characterization of the mouse analogues of CD59 using novel monoclonal antibodies: Tissue distribution and functional comparison. *Immunology* 109(1):117–126.
45. Ruseva MM, Heurich M (2014) Purification and characterization of human and mouse complement C3. *Methods Mol Biol* 1100:75–91.