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Targeted complement inhibition salvages stressed neurons and inhibits neuroinflammation after stroke in mice

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Abstract

Ischemic stroke results from the interruption of blood flow to the brain resulting in long-term motor and cognitive neurological deficits, and it is a leading cause of death and disability. Current interventions focus on the restoration of blood flow to limit neuronal death, but these treatments have a therapeutic window of only a few hours and do not address post-stroke cerebral inflammation. The complement system, a component of the innate immune system, is activated by natural immunoglobulin M (IgM) antibodies that recognize neoepitopes expressed in the brain after ischemic stroke. We took advantage of this recognition system to inhibit complement activation locally in the ischemic area in mice. A single chain antibody recognizing a post-ischemic neoepitope linked to a complement inhibitor (termed B4Crry) was administered systemically as a single dose after stroke and shown to specifically target the ischemic hemisphere and improve long-term motor and cognitive recovery. We show that complement opsonins guide microglial phagocytosis of stressed but salvageable neurons, and that by locally and transiently inhibiting complement deposition, B4Crry prevented phagocytosis of penumbral neurons and inhibited pathologic complement and microglial activation that otherwise persisted for several weeks after stroke. B4Crry was protective in adult, aged, male and female mice and had a therapeutic window of at least 24 hours after stroke. Furthermore, the epitope recognized by B4Crry in mice is overexpressed in the ischemic penumbra of acute stroke patients, but not in the contralateral tissue, highlighting the translational potential of this approach.

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Competing interests: S.T. is an inventor on a patent application for natural antibody-targeted complement inhibitors filed by the University of Colorado (PCT/US2014/012831; Targeting constructs based on natural antibodies and uses thereof) and is a consultant for Admirx Inc., a company developing complement inhibitors. He also holds stock from Admirx Inc.

INTRODUCTION

The complement system is a component of innate immunity that is activated on the surface of pathogens and stressed cells (1). Once activated, complement is capable of self-amplification and can trigger an inflammatory response and modulate both innate and adaptive immune processes (1). Ischemic stroke is associated with pathologic activation of complement in the ischemic brain leading to deposition of complement opsonins [complement components 1q (C1q) and 3d (C3d) and mannose binding lectin] and release of complement anaphylatoxins (C3a and C5a) (2). Although complement is implicated in promoting post-stroke pathology and worsening of outcome, little is known concerning the mechanism of complement-dependent acute and chronic neurodegeneration after stroke (2, 3). After stroke, complement activation products contribute to the recruitment and activation of immune cells, especially microglia, but also appear to play a role in recovery mechanisms by promoting subsequent resolution of inflammation and regeneration (4). This dual role of complement in injury and recovery provides a challenge for the application of complement-targeting strategies to treat stroke [reviewed in (2)].

In mouse models, complement activation induced by ischemic stroke is triggered by the binding of self-reactive natural immune-globulin M (IgM) antibodies to ischemia-induced damage-associated molecular patterns (DAMPs), or neopeptides, expressed on the surface of stressed and dying cells (5). We have previously shown that whereas $Rag1^{-/-}$ mice, which inherently do not have antibodies, are protected against cerebral ischemia and reperfusion injury, reconstitution of $Rag1^{-/-}$ animals with an IgM monoclonal antibody (mAb) (B4) that recognizes a post-stroke modified annexin IV neopeptide restores cerebral injury (6). Here, we characterize a strategy for complement inhibition that is targeted specifically to sites of modified annexin IV expression and that exploits the natural IgM and complement dangersensing system (5, 7) to provide localized and transient inhibition of complement in the post-ischemic brain. A single chain antibody (scFv) derived from B4 IgM mAb (B4scFv) was linked to murine CRRY, an ortholog of human complement receptor 1 (CR1) that inhibits all complement pathways at the C3 activation step (7). The fusion construct, called B4Crry, as well as B4scFv alone, were used here as therapeutic agents and to investigate how IgM and complement activation are involved in shaping the cerebral inflammatory phenotype after ischemic stroke in a clinically relevant paradigm.

RESULTS

B4Crry targets post-ischemic cells and inhibits IgM binding and complement activation

We first characterized B4Crry in an in vitro model of oxygen-glucose deprivation in murine brain endothelial cells (bEnd.3) and neuronal cells (Cath.a). After oxygen-glucose deprivation and reoxygenation of bEnd.3 cells, B4Crry bound to hypoxic but not normoxic cells, inhibited binding of the parent B4 mAb, and inhibited complement activation (C3d deposition) (fig. S1, A to C). In addition, oxygen-glucose deprivation and reoxygenation of Cath.a cells resulted in robust deposition of B4-IgM that was inhibited when cells were co-incubated with B4Crry (fig. S1, D and E).

We next assessed the duration and injury specificity of the B4-targeted neoepitope after transient middle cerebral artery occlusion (MCAO) in mice by biodistribution studies using radiolabeled antibodies administered via tail vein injection. B4 IgM mAb, but not control F632 IgM mAb, specifically targeted the ipsilateral (right) hemisphere with minimal localization of either mAb in the contralateral hemisphere or other organs (fig. S2, A to C). We also demonstrated that radiolabeled B4Crry administered 2 hours after MCAO showed specific targeting to the ipsilateral hemisphere with minimal localization in other organs and no brain localization in sham animals (Fig. 1A). We showed higher specific activity of B4Crry in the ischemic brain when administered at 24 hours after MCAO compared to 2 hours (Fig. 1A). This is likely due to increased blood-brain barrier (BBB) permeability given that a positive correlation was found between the specific activity of B4Crry in the ischemic brain and the extent of BBB permeability (fig. S2, D and E; $r^2 = 0.602$, $P < 0.001$). We previously demonstrated that B4Crry has a short circulatory half-life (fast-phase $T_{1/2}$ of 9.7 min and second-phase $T_{1/2}$ of 5.5 hours) (7), and here, we demonstrated that the tissue half-life in the brain after stroke was about 35 hours (fig. S2F). A short circulatory half-life and prolonged tissue half-life are features intentionally designed to provide benefit of complement inhibition locally with minimal impact on systemic complement activity. We further show that B4Crry inhibited complement at its target site, as indicated by reduced production of the activation products C3a and C3d in the ipsilateral hemisphere (Fig. 1, B and C). B4Crry administered at a dose that was effective at inhibiting complement in the brain did not affect systemic serum complement activation and did not affect serum complement activity in either sham or stroke mice (fig. S2, G and H).

To further confirm the targeting specificity of B4Crry after stroke, we intravenously administered B4Crry, tagged with a histidine repeat sequence for in situ detection, to sham and stroke mice 2 hours after surgery. IF staining of brains extracted 2 hours after administration showed robust binding of B4Crry to the ipsilateral cortex and hippocampus of stroke but not sham animals (fig. S3A). Localization of B4Crry to the ipsilateral brain parenchyma was confirmed by high-resolution imaging showing binding of B4Crry to the membrane of neurons in the perilesional cortex and hippocampus at 2 hours after injection, with minimal binding seen in the contralateral brain (fig. S3B). Neurons recognized by B4Crry also expressed c-fos (fig. S3B), a marker of neuronal activity that is also up-regulated in the event of stress (8, 9). These findings are supported by in vitro data showing B4Crry binding to a neuronal cell line after exposure of cells to hypoxia (fig. S1E). Ischemic insult affects endothelial cells in addition to the brain parenchyma, and we showed that B4Crry colocalized with the endothelial marker CD31 in the ipsilateral hemisphere (fig. S3C). These data indicate that B4Crry binds to ischemic cerebral vasculature after stroke and gains access to cerebral parenchyma likely due to loss of BBB integrity.

B4scFv and B4Crry have distinct temporal effects on complement activation and post-stroke pathology

The natural IgM antibody (Ab) repertoire consists of self-reactive Abs that bind to multiple post-ischemic neoepitopes (10). Here, we assessed how stroke pathology is influenced by the complete absence of the IgM Ab repertoire, as well as by the specific blockade of the annexin IV neoepitope with B4scFv. We also investigated the effect of combined annexin IV

neopeptide blockade and complement inhibition with B4Crry. Rag1^{-/-} mice and both B4scFv- and B4Crry-treated WT mice all showed similar improvement in neurological deficits and reduced infarct volume 24 hours after stroke compared to vehicle-treated WT mice (Fig. 1, D and E). These improvements were accompanied by a reduction in IgM and C3d deposition (Fig. 1C and fig. S4, A and B). However, B4scFv alone did not interrupt the evolution of acute infarct, and neurological scores worsened by day 3 (Fig. 1D). Worsened out-comes in B4scFv-treated mice 3 days after stroke were associated with a parallel increase in IgM binding and complement activation (fig. S4, C and D). On the contrary, B4Crry treatment showed sustained reduction in infarct volume and neurological deficits after 3 days (Fig. 1D) associated with prolonged complement-inhibitory effect and reduced IgM deposition (fig. S4, C and D). Thus, blocking a single specificity of natural IgM is not sufficient for sustained inhibition of cerebral injury, and delivery of a complement inhibitor is required for sustained protection (3, 6). B4Crry administration to C3^{-/-} animals 2 hours after MCAO did not provide additional protection to C3^{-/-} mice in terms of reduction of infarct volume or neurological deficit (fig. S5, A to C), indicating that B4Crry therapeutic activity is dependent on C3.

Post-stroke chronic recovery is improved after local and transient complement inhibition by B4Crry

We next followed mice through 15 days of recovery, with B4Crry intravenously administered at either 2 or 6 hours after MCAO. B4Crry-treated animals had lower acute neurological deficits and smaller lesion size compared to vehicle-treated controls and showed a greater improvement in recovery of initial deficit over the 15-day period (Fig. 2A). Lesion reconstruction and mapping (see Supplementary Materials and Methods and fig. S6) showed injury with secondary astrogliosis in ipsilateral basal ganglia, cortex, and hippocampus in vehicle-treated mice (Fig. 2, B and C, and fig. S7). Both cortical and hippocampal involvement was minimal in mice treated with B4Crry, which substantially reduced infarct volume at 15 days (Fig. 2C and fig. S7). In agreement with these histopathological findings, B4Crry treatment increased locomotor activity in open field and reduced laterality on corner test and pasta handling task (Fig. 2, D to F). There were no differences in the above outcome measures whether B4Crry was administered at 2 or 6 hours after MCAO.

To investigate whether hippocampal protection by B4Crry is functionally relevant, we assessed the retention of fear memory learned before stroke using the passive avoidance task as well as the spatial learning and memory after stroke using Barnes maze task. Animals treated with B4Crry had higher memory retention on passive avoidance compared to vehicle-treated mice 3 to 12 days after stroke (Fig. 2G). Spatial learning was also improved by the treatment; animals treated with B4Crry had faster learning curves and better retention of learned memory on Barnes maze (Fig. 2H).

Learning after stroke can be partially dependent on the regenerative processes that occur during chronic recovery (11). Doublecortin (Dcx) is expressed on neuronal precursor cells and immature neurons and serves as a marker for neurogenesis. We therefore investigated the incorporation of Dcx⁺ neuroblasts into the ipsilateral hippocampus and cortex 15 days

after injury; B4Crry-treated animals had a four-fold increase in the number of cortical and hippocampal Dcx⁺ neuro-blasts compared to vehicle (Fig. 2, I and J). Thus, B4Crry can prevent the evolution of secondary injury not only by reducing acute injury but also by promoting recovery mechanisms.

Neuroprotective effects of B4Crry are consistent across gender, age, and temporal variables

We next demonstrated that B4Crry administration 2 hours after MCAO exerted therapeutic effects on neurological deficits, infarct volume, forearm asymmetry, and mortality for at least 30 days after reperfusion (Fig. 3, A to D). In accordance with the Stroke Treatment Academic Industry Roundtable (STAIR) recommendations (12), we also investigated B4Crry treatment across different ischemia times (Fig. 3, E and F) and across gender (Fig. 3, G and H) and in aged (10 months old) mice (Fig. 3, I and J). Neuroprotection was maintained across each of the different variables in terms of a reduction in neurological deficit scores and infarct volume compared to vehicle when B4Crry was administered intravenously 2 hours after MCAO (Fig. 3, E to J). Moreover, B4Crry administered 2 hours after MCAO also improved survival of aged animals (Fig. 3J). When the administration of B4Crry was delayed to 24 hours after MCAO in adult mice, B4Crry reduced neurological deficits and forearm laterality, improved memory retention on Barnes maze, and reduced infarct volume compared to vehicle-treated controls (Fig. 3, K to N). This delayed treatment window is a marked improvement over the 3- to 4.5-hour clinical window for tissue plasminogen activator treatment, the only currently approved pharmacological agent for stroke therapy (13).

We have previously reported an alternative strategy for targeting CRRY and other complement inhibitors that involves linking a complement inhibitor to complement receptor 2 (CR2Crry) (14). Unlike B4Crry, CR2Crry did not improve neurological deficit or improve cognitive performance compared to vehicle-treated controls as measured 15 days after MCAO (fig. S8, A and B).

Complement inhibition by B4Crry does not increase susceptibility to infection in a model of murine pneumonia

One theoretical advantage of using ischemia-specific targeted complement inhibitors is the absence of systemic complement inhibition that may predispose to infection, a major cause of morbidity and mortality in stroke patients (15). We investigated the effect of B4Crry on mortality in a murine model of infectious pneumonia (16, 17). Whereas intranasal administration of *Streptococcus pneumoniae* resulted in an increase in mortality in C3^{-/-} mice (100% mortality within 2 days) compared to WT mice (~60% mortality by day 4), B4Crry did not increase mortality rate in infected animals (fig. S9). These data highlight an important advantage of targeted versus systemic inhibition of complement for the treatment of stroke.

Complement activation promotes neuronal stress and triggers microglial phagocytosis of live neurons

We hypothesized that by preventing pathologic complement activation, B4Crry breaks a self-amplified cascade of acute inflammation and microglial activation and limits propagation of chronic neuro-inflammation. Because the extent of initial injury and neuronal loss is a major predictor of chronic recovery and response to rehabilitation therapy in stroke patients (18), we examined the perilesional brain areas of animals treated with vehicle or B4Crry administered 2 hours after ischemia. Compared to vehicle-treated animals, there was about two times higher density of neuronal nuclear antigen (NeuN)-expressing neurons in the peri-infarct area of B4Crry-treated animals at 24 hours after reperfusion (Fig. 4, A and B). In addition, compared to vehicle-treated animals, both B4Crry-treated animals and C3-deficient animals had a lower density of c-fos⁺ neurons, a marker of cellular stress and high calcium influx that specifically localizes to live neurons that do not express the apoptotic marker cleaved caspase-3 (Fig. 4, C to F and fig. S10A). B4Crry-treated animals and C3-deficient animals also showed a lower density of Iba1⁺ microglia compared to vehicle controls (Fig. 4, C, D, and G); Iba1 is a microglia/macrophage marker that is up-regulated in activated microglia. This supports the hypothesis that inhibition of complement activation suppresses the activation of perilesional microglia and reduces cellular stress in perilesional neurons.

Next, we assessed whether complement deposition after stroke may potentiate perilesional neuronal loss by promoting microglia-dependent clearing of stressed, but live penumbral neurons (19,20). We used c-fos to label stressed neurons. Co-staining with Iba1 and c-fos showed reduced colocalization of c-fos material inside microglia cell bodies in B4Crry-treated mice (Fig. 4, H and I) compared to vehicle-treated controls. Animals treated with B4scFv did not show a reduction in c-fos⁺ cell density, Iba1⁺ cell density, or c-fos material localization to microglia (Fig. 4, E, G, and H). Localization of c-fos material and attraction of microglia to c-fos⁺ neurons (NeuroTrace⁺) was also observed in the ipsilateral hippocampus of vehicle controls (fig. S10, A and B). Supporting our finding that microglia are attracted to c-fos⁺ neurons in vehicle controls, but not in B4Crry-treated animals, we observed a positive correlation between the density of Iba1⁺ and c-fos⁺ cells per field in vehicle controls that was absent with B4Crry treatment (fig. S10, C and D). These findings indicate that inhibiting complement deposition prevents the attraction of perilesional microglia to live neurons and prevents the phagocytosis of neuronal material (movies S1 to S4).

To further confirm the presence of neuronal material within Iba1⁺ cells, we demonstrated colocalization of NeuN material within Iba1⁺ cells from vehicle-treated and B4scFv-treated mice (Fig. 4J). In contrast, B4Crry-treated mice showed an absence of neuronal material localized within Iba1⁺ cells (Fig. 4J). To correct for overall increase in microglial density in vehicle controls, we also show that B4Crry reduced the proportion of Iba1⁺ cells with c-fos material (Fig. 4K). We then used a marker of activated and proliferating microglia [Mac2 (21)] and a microglia-specific marker [Fc receptor-like S (FCRLS) (21, 22)] to demonstrate that the cells harboring c-fos material are activated resident microglia (Fig. 4, L to O). B4Crry treatment reduced the number of proliferating (Mac2⁺) microglia and reduced c-fos⁺

localization to Mac2⁺ microglial cell bodies (Fig. 4, K to M). We also demonstrated that Iba1⁺ cells harboring c-fos material were also FCRLS⁺ indicating microglial nature (Fig. 4, N and O, and fig. S11A). At 3 days after reperfusion, IF staining of the ipsilateral hemisphere showed the continued presence of c-fos material within the cytosol of brain resident microglia in vehicle controls, but not in B4Crry-treated animals (fig. S11, B and C).

We also administered B4Crry 6 hours after reperfusion to assess whether inhibition of complement abolishes microglial phagocytosis of neurons after a longer duration of perilesional insult. Compared to 2-hour administration of B4Crry, animals treated 6 hours after MCAO showed a higher density of c-fos⁺ cells in the perilesional brain at 24 hours after insult. However, the treatment was still able to reduce the colocalization of perilesional microglia to stressed neurons and to lower the frequency of c-fos material in microglia compared to vehicle controls (fig. S12, A to C).

Complement opsonins tag stressed, but live neurons for microglial phagocytosis

We next assessed whether C3 opsonins are responsible for microglial association and subsequent phagocytosis of c-fos⁺ neurons. Although activated microglia may contribute to the phagocytosis of different cells and debris after stroke, we focused on the interaction between microglia, complement, and neurons. IF staining showed that the C3d deposition at the microglial interface with c-fos⁺ neurons observed in vehicle-treated animals (Fig.5A) was inhibited by B4Crry treatment (Fig.5B). Co-staining with the neuronal marker NeuN showed that c-fos⁺ cells tagged by C3d and surrounded by microglia are also NeuN⁺, confirming their neuronal identity (Fig. 5C and movie S5).

To quantify C3d deposition at microglial-neuronal interfaces, we determined the percentage of neurons that are C3d⁺ and/or c-fos⁺ among the total number of Iba1⁺-NeuN⁺ interfaces (Fig.5D). Treatment with B4Crry reduced the proportion of C3d⁺ microglia-neuron interactions (Fisher exact test, $P < 0.001$), with C3d deposited on ~77% of microglia-neuron interfaces in vehicle controls compared to ~6% in B4Crry-treated animals (Fig. 5D). In vehicle controls, most of the C3d⁺ neurons were also c-fos⁺, indicating preferential complement deposition on c-fos⁺ neurons (Fig.5D). These findings were further validated by flow cytometry showing a reduction in C3d deposition on neurons and c-fos⁺ cells in B4Crry and C3^{-/-} animals compared to controls 24 hours after MCAO (fig. S13). We also investigated whether the soluble complement activation products C3a and C5a may play a role in complement-directed microglial destruction of neurons, because these anaphylatoxins can recruit and activate immune cells including microglia [reviewed in (2, 3)]. Deficiency of C3a or C5a receptors in mice subjected to MCAO did not reduce the number of c-fos⁺ neurons and did not reduce the number of Iba1⁺ cells compared to vehicle controls 24 hours after MCAO (fig. S13). Collectively, these data indicate that neuronal phagocytosis of stressed neurons in the ischemic brain occurs acutely after stroke, that the process is complement opsonin-dependent, and that it can be inhibited by blocking C3 cleavage with B4Crry.

To confirm neuronal phagocytosis by inflammatory microglia, we used staining of the lysosomal marker LAMP1 to track early phagolysosomal vesicles. Both c-fos material and NeuN material were found to colocalize to LAMP1⁺ clusters within microglia in vehicle

controls (fig 5, E and F, and movies S6 and S7). We also quantified the number of triple-positive clusters (Iba1⁺, c-fos⁺, and LAMP1⁺) using the peaks of intensity histograms shown in Fig. 5G, and demonstrated a reduction in the number of triple-positive clusters in B4Crry-treated mice compared to vehicle controls (Fig. 5H).

B4Crry does not inhibit clearance of degenerating cells and debris

Microglia and complement have also been implicated in reparatory mechanisms after stroke, and microglial depletion has a negative influence on post-stroke outcomes and prevents restoration of neuronal activity (23). In this regard, B4Crry did not completely block ipsilateral microglial activation after MCAO, and a higher density of Iba1⁺ cells was still observed in the ipsilateral hemisphere of B4Crry-treated mice compared to the contralateral hemisphere (fig. S14A). However, this increase was reduced compared to vehicle-treated animals, and Iba1⁺ cells in B4Crry-treated animals were predominantly of branched rather than amoeboid (activated) morphology (fig. S14A). B4Crry treatment resulted in a decrease in Fluoro-Jade B-labeled degenerating neurons on day 3 compared to day 1 after stroke (fig. S14B), suggesting intact apoptotic and dead cell clearance machinery. These data are in accordance with the lack of effect of B4Crry on upstream C1q deposition and on microglial targeting of apoptotic caspase-3⁺ cells in the ipsilateral brain, which is an important mechanism of apoptotic cell clearance in the central nervous system (CNS) (fig. S14, C and D).

B4Crry limits the propagation of chronic neuroinflammation and neurodegeneration

To investigate whether complement inhibition prevents the propagation of neuroinflammation during recovery, we performed high throughput NanoString gene expression analysis of 561 immunology-related genes 5 days after MCAO (fig. S15), a time point at which B4Crry has been largely cleared from the brain (fig. S3F). Several differentially expressed genes that were down-regulated by B4Crry belonged to interferon- γ (IFN- γ) signaling, cytokine signaling, locomotion, and complement activation pathways (Fig. 6, A and B). B4Crry down-regulated expression of *C3* and components of alternative (*factor B*) and classical (*C1q*) pathways (Fig. 6C). A large panel of microglial activation and attraction factors was also down-regulated by B4Crry (Fig. 6D). A few genes were up-regulated with B4Crry treatment; these genes were not enriched for specific biological processes but included the complement inhibitor *CD55* and antiapoptotic *Bcl-2* genes (fig. S15C). Follow-up construction and analysis of the protein-protein interaction network using Ingenuity Pathway Analysis demonstrated that B4Crry treatment resulted in a down-regulation of key modulators of microglial proliferation and activation, including *IFN- γ* , IFN regulatory factor-8, nuclear factor κ light-chain enhancer of activated B cells, and signal transducer and activator of transcription 3 (fig. S15D), suggesting that B4Crry dampened the over all inflammatory response.

We next assessed the relevance of these findings in relation to microglial activation during long-term recovery, as well as the effect of acute B4Crry treatment on chronic microgliosis. Mac2⁺ microglia/macrophages are indicative of microgliosis, and their presence suggests the continuous occurrence of a noxious or inflammatory insult. Using immunostaining of full-brain sections for Mac2, we demonstrated that vehicle controls developed robust

microgliosis that covers around 20% of the ipsilateral hemisphere (Fig. 6, E and F). Post-stroke microgliosis persisted for at least 30 days after stroke in vehicle-treated mice (fig. S16). In contrast, microgliosis was limited to areas around the occlusion site in B4Crry-treated animals. M2-like microglia/macrophages, identified by the marker Ym1, are known as alternatively activated and consist of a continuum of functional states associated with a T helper 2 cell immune response and, in general, a more anti-inflammatory profile (24). B4Crry-treated animals showed very limited microgliosis and a higher ratio of Ym1⁺/Mac2⁺ microglia, indicating higher abundance of alternatively activated microglia (Fig. 6, E and F). Compared to acute microglial activation, at 15 days after MCAO, control mice had more than a four-fold increase in density of Iba1⁺ cells in the ipsilateral hemisphere, whereas B4Crry-treated mice did not show an increase in Iba1⁺ cells between days 1 and 15 (fig. S16, A and B).

Although the extent of initial injury is a major determinant of chronic outcomes after stroke, post-stroke remodeling and regeneration may further contribute to recovery. Conversely, chronic neuroinflammation is associated with reduced post-stroke neuroplasticity and poor motor and cognitive outcomes (25). We investigated the relationship between the chronic inflammatory response and neurodegeneration 15 days after stroke by assessing the density of microglia, neurons, and dendrites in the perilesional brain, defined using stereological coordinates and quantified as described in fig. S17. At 15 days after injury, compared to vehicle controls, animals treated with B4Crry had a higher dendritic and neuronal density, a lower microglial density, and a higher microglial branching index (Fig. 5, G to I). These data indicate that ongoing microgliosis in the perilesional brain is associated with loss of neurons and dendrites, reflecting a neurodegenerative process that is inhibited by B4Crry. We observed a negative correlation between the number of proliferating (Mac2⁺) microglia and the number of neurons per field ($r^2 = 0.6$, $P < 0.001$; fig. S18).

B4Crry prevents sustained long-term complement deposition after stroke

We next assessed both the extent and the pattern of complement deposition 15 days after stroke. Two distinct patterns were observed in the perilesional brain. At the edge of the ischemic core, robust extravascular C3d deposition was present in areas of microgliosis in vehicle controls (Fig. 7A), whereas minimal C3d deposition was found in B4Crry-treated mice (Fig. 7B). Microglial migration was also found to target sites of C3d deposition (Fig. 7A, inset 2). In addition, a distinct pattern of complement activation was observed in the perilesional cortex and hippocampus 15 days after injury, with predominant vascular localization as assessed by colocalization with the endothelial cell marker CD31 (Fig. 7C). Perilesional C3d deposition was also associated with deposition of IgM, and both C3d and IgM deposition was inhibited by B4Crry treatment (fig. S19). We also demonstrated that endothelial deposition of complement in vehicle controls was associated with increased immune cell infiltrate into the ischemic brain 15 days after stroke, because vehicle controls showed a higher number of CD3⁺ T cells and Gr1⁺ neutrophils in the ipsilateral hemisphere compared to B4Crry-treated mice (Fig. 7, D to F). These findings indicate that chronically sustained complement activity in the ischemic hemisphere is associated with robust microgliosis, persistent neurodegeneration, and increased inflammatory cellular infiltrates.

Early targeted inhibition of complement interrupted this neuroinflammatory degenerative response.

A similar B4 mAb-specific DAMP is expressed in post-ischemic human brains

Finally, we investigated whether there is a similar B4 recognition system in humans. We used B4scFv, B4Crry, and the parent B4 IgM mAb to probe postmortem brain sections from the penumbra of patients who died from acute stroke. There was extensive binding of B4scFv to the ischemic penumbra, with a similar pattern of binding seen in human and murine ischemic brains 24 hours after stroke. Minimal binding of B4scFv was detected on sections from the contralateral hemisphere from stroke patients (fig. S20, A and B). We analyzed samples from three stroke patients who died 24 to 72 hours after stroke onset, and B4scFv binding was observed in the ischemic penumbra at all time points (fig. S20C). Similarly, B4Crry (fig. S21A) and B4 IgM mAb (fig. S21B), but not a control IgM mAb (D5) (fig. S21C), bound to sections prepared from the ischemic penumbra, but not to sections from the contralateral hemisphere.

DISCUSSION

The recently described roles of complement in sculpting neuronal circuitry during development and in promoting synaptic and neuronal loss during neurodegenerative disease have revealed key insights into complement-microglial interactions (26). However, little is known about complement-microglial interactions in diseases of acute cerebral insult where complement pathways contribute to both neurodegenerative and neuroregenerative processes (2). We used a strategy of targeted complement inhibition to modulate complement specifically at the site of injury (fig. S22). We show that after cerebral ischemia, complement activation promotes neuronal stress and reduces the probability that stressed neurons will recover by tagging live neurons for uptake by microglia. Stressed, but still live neurons in the ischemic penumbra display danger-associated molecular patterns that lead to deposition of C3d on their membranes in an IgM- dependent mechanism. C3 opsonization results in subsequent rapid clearance of stressed neurons by inflammatory microglia. This neurodegenerative response accounts for the rapid loss of neuronal reservoir in the ischemic penumbra and promotes a proinflammatory milieu that drives chronic neurodegenerative inflammation. We demonstrate that this pathologic complement-microglial interaction is sustained for at least a month after murine stroke and that, by transiently inhibiting complement activation, sustained complement deposition and microgliosis is suppressed, limiting the loss of neurons and neuronal connections.

A concern in the application of complement-inhibitory strategies is the impact of complement modulation on systemic homeostatic processes, as well as reparatory and remodeling processes that tend to occur beyond the acute phase of injury (14, 27). In this context, we demonstrated that the transient nature and ischemia-specific targeting of complement inhibition with B4Crry did not adversely affect reparatory processes. Also, B4Crry did not interrupt microglial clearance of debris and apoptotic cells, an important finding given that microglial depletion results in worsening of outcomes in models of acute CNS injury (23). Infection is a frequent post-stroke complication, and we also show that

unlike C3 deficiency, B4Crry treatment does not affect susceptibility to infection by *S. pneumoniae*. In addition, B4Crry showed efficacy when administered to mice 24 hours after stroke onset, and treatment did not carry the risk of intracerebral hemorrhage, a major advantage over thrombolytic therapy.

Another report of targeted complement inhibition in cerebral ischemia reperfusion injury was from our laboratory and involved linking CR2 to a complement inhibitor (CR2Crry) (15). However, CR2Crry did not maintain protection through the subacute phase after stroke in the same MCAO paradigm used here and inhibited regenerative processes (14). Unlike B4scFv, CR2 targets all sites of C3 deposition, including sites of non-ischemia-related complement activity that may be important for optimal recovery; in addition, B4scFv targets a more proximal point in the complement-mediated inflammatory pathway and may also contribute to therapeutic activity by competing with pathogenic IgM binding, at least in the acute phase.

The human ortholog of murine CRRY is CR1, with the two proteins having similar structural and activity profiles (28). Soluble CR1 has been investigated in clinical trials (29), and a previous report investigating only acute outcomes after murine stroke described the localized delivery of soluble CR1 to the site of cerebral ischemia via P-selectin binding using sialyl-Lewis^x-tagged soluble CR1. This tagged CR1 molecule was more effective at protecting from acute outcomes compared to untagged soluble CR1 (30), although the efficacy of the targeted inhibitor was still dependent on systemic complement inhibition (2, 30).

To conclude, we note that there are limitations to this study. We have not specifically addressed how complement inhibition affects the vasculature. It is likely that B4Crry also affects the activation of endothelial or perivascular cells and the transfer of blood proteins and cells to the injury site, both of which may contribute to the protective effects of B4Crry. B4Crry also influences anaphylatoxin generation; in the present study, we did not address the role of the anaphylatoxins outside of demonstrating that they did not affect neuron phagocytosis after stroke. Whether targeting alternative post-ischemic neoepitopes, individually or together, will provide better protection than B4scFv targeting is unknown, as is the cross-species conservation of other injury-specific neoepitopes. Nevertheless, we show that the B4 epitope is expressed in both mouse and human post-ischemic brains and that B4Crry treatment protected against murine ischemic stroke even when administered 24 hours after MCAO, a clinically relevant therapeutic window.

MATERIALS AND METHODS

Study design

The overall study design is shown in fig. S22A. For all studies, before their initial acclimation on behavior tasks, animals were randomly assigned to treatment groups using a random number generator. Lab personnel involved in surgeries, testing, and scoring were blinded to group allocations for the duration of the study. Animals were excluded only if mortality occurred during surgery, before surgery, or before administration of treatment (<10% of animals). End points were determined by time after reperfusion and were 24

hours, 72 hours, 15 days, or 30 days after MCAO, or for humane reasons defined by institutional guidelines. For acute studies (fig. S22B), end points were determined as mortality of animals or reaching the end point of 24 or 72 hours after MCAO, depending on the experiment. For long-term studies (fig. S22B), a 15- or 30-day time point was used to evaluate the dynamics of neuroprotection according to STAIR recommendations (evaluation of outcomes in preclinical stroke at least 14 days after injury).

All analyses were performed with the last observation carried forward for animals that drop out because of post-stroke mortality beyond day 1 of reperfusion. Single animals (not fields in histological studies) were considered a unit for statistical analyses. Sample size determination was performed using G*Power 3.1 (31). Effect size for behavioral analyses was determined on the basis of pilot experiments with B4Crry, and calculations were made on the basis of the following criteria: power of 80% and a significance level <0.05 (corrected for multiple comparisons wherever appropriate).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad). Parametric testing was used unless otherwise specified. Neurological deficit scores were compared using nonparametric *t* tests. Group analyses were performed using one-way ANOVA (or two-way ANOVA for multiple time points) followed by multiple comparison analyses or using repeated-measures ANOVA for matched data. *P* values below 0.05 were considered significant. Brown-Forsythe test was used to assess for homogeneity of variance, and Kruskal-Wallis test was used for nonparametric testing. Student's *t* test was used to compare two groups and was always used as two-tailed. Pearson correlation coefficients were used to compute correlations, and Fisher *r*-to-*z* transformation was used to compare correlation coefficients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Systemically administered B4Crry locally and transiently inhibits complement activation in the post-ischemic brain and reduces acute injury.

Experiments performed in adult wild-type (WT) male mice treated with vehicle, B4scFv, or B4Crry or in $C3^{-/-}$ mice (as indicated in figure) that underwent 1-hour MCAO followed by reperfusion. **(A)** Biodistribution of ^{125}I -radiolabeled B4Crry administered at 2 or 24 hours after MCAO and measured at 6 hours after administration in MCAO or sham animals. Two-way analysis of variance (ANOVA) with Bonferroni, $n = 4$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. **(B)** C3a concentration measured by enzyme-linked immunosorbent assay (ELISA) on brain homogenates from sham animals or MCAO animals treated with B4Crry or vehicle 2 hours after stroke. ANOVA with Bonferroni, $n = 3$ animals, $*P < 0.05$ and $***P < 0.001$. **(C)** Immunofluorescence (IF) staining of C3d deposition in the ischemic hemisphere 24 hours after stroke. B4Crry was administered 2 hours after MCAO. Scale bars, 20 μm . **(D)** Infarct volume measured by tetraphenyl tetrazolium chloride (TTC) staining of 2-mm-thick sections at 24 or 72 hours after stroke in WT animals treated with vehicle, B4scFv, or B4Crry and in $\text{Rag1}^{-/-}$ mice. Treatments were administered 2 hours after MCAO, $n = 8$ per group (5 for $\text{Rag1}^{-/-}$). ANOVA with Bonferroni, $*P < 0.05$ and $**P < 0.01$. **(E)** Neurological deficit scores at 24 or 72 hours after stroke. Kruskal-Wallis test, $*P < 0.05$.

Fig. 2. B4Crry administered acutely after MCAO improves long-term histopathological, motor, and cognitive recovery.

Experiments performed in WT animals subjected to 1-hour MCAO and treated with vehicle, B4scFv, or B4Crry at 2 hours after MCAO or with B4Crry at 6 hours after MCAO. (A) Daily neurological deficit score collected throughout 15 days of recovery. Repeated-measures two-way ANOVA with Bonferroni, $n = 9$ [B4scFv and B4Crry (6 hours)] and $n = 13$ [vehicle and B4Crry (2 hours)], $^{***}p < 0.001$ comparing vehicle to B4scFv and B4Crry (2 hours) and $^{***}P < 0.001$ comparing vehicle to B4Crry (2 hours) and B4Crry (6 hours). (B) Infarct volumes reconstructed from serial Nissl-stained sections (200 μm apart) harvested 15 days after MCAO. One-way ANOVA with Bonferroni, $n = 9$ per group, $*P < 0.05$ and $^{****}P < 0.001$. (C) Lesion was reconstructed as described in fig. S6, and three-dimensional (3D) models show the lesion in red mapped to the ipsilateral hemisphere, the cortex in yellow, basal ganglia in green, and hippocampus in blue. A coronal cut was performed at Bregma +0.38, and cross-sectional views are shown to the right of 3D models. (D to F) Motor recovery measured by open-field locomotion (D), forelimb laterality (corner task, E), and pasta handling (F) over 15 days of recovery. Two-way ANOVA with Bonferroni, $n = 8$ animals per group and $n = 12$ animals per group for corner task vehicle and B4Crry (2 hours); $*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (G and H) Passive avoidance (G) and Barnes maze (H) tasks. Two-way ANOVA, $n = 8$ (Barnes), $n = 8$ [avoidance controls and B4Crry (6 hours)], and $n = 11$ [avoidance B4Crry (2 hours)]; $*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to controls (vehicle). (I) IF staining for Dcx⁺ neuroblasts migrating to the peri-infarct hippocampus and cortex 15 days after MCAO. Images represent 3D

reconstruction of 20- μm z-stack. Scale bars, 20 μm . DAPI, 4',6-diamidino-2-phenylindole. (J) Quantification of (I). Multiple t tests with Holm-Sidak correction, $n = 5$ animals (three fields per animal), * $P < 0.05$ and ** $P < 0.01$. Data are reported as means $\pm$ SEM in all panels.

Fig. 3. B4Crry provides neuroprotection across different ischemia times, gender, and age and with delayed administration.

Experiments performed in vehicle- or B4Crry-treated adult male mice that underwent 1-hour MCAO followed by 30 days of reperfusion. (A) Neurological deficit assessment performed over 30 days. Two-way ANOVA with Bonferroni, $n = 9$ (vehicle) and $n = 6$ (B4Crry), $**P < 0.01$. (B and C) Infarct volume calculated from serial brain sections stained with Nissl (B) and forelimb laterality on corner task (C) 30 days after reperfusion. Student's t test, two-tailed; $n = 9$ (vehicle) and 6 (B4Crry), $*P < 0.05$ and $***P < 0.001$. (D) Kaplan-Meier plot

of survival over 30 days after stroke. Log-rank (Mantel-Cox) test, $*P < 0.05$. **(E and F)** Experiments performed in adult male mice with variable ischemia time, and outcomes assessed 24 hours after reperfusion. **(E)** Neurological deficit scores at 24 hours after MCAO. Multiple t tests with Holm-Sidak correction, $n = 7$ (30 and 90 min) and $n = 13$ (60 min), $\#P = 0.06$ and $*P < 0.05$. **(F)** Infarct volume calculated from serial brain sections stained with Nissl 15 days after MCAO. Student's t test, $n = 6$ per group, $*P < 0.05$ and $^{\$}P = 0.06$. **(G and H)** Experiments performed in adult female mice with 1-hour MCAO followed by 15 days of reperfusion. B4Crry/vehicle administered 2 hours after ischemia. **(G)** Neurological deficit assessed throughout recovery. Two-way ANOVA, $n = 48$ (vehicle) and $n = 7$ (B4Crry), $*P < 0.05$ and $**P < 0.01$. **(H)** Infarct volume assessed by Nissl staining 15 days after MCAO. Student's t test, $n = 7$, $*P < 0.05$. **(I)** Neurological deficit and infarct volumes (measured by TTC) in aged male mice (10 months old) at 24 hours after 1-hour MCAO and B4Crry administration 2 hours after MCAO. Mann-Whitney test for neurological deficit and Student's t test for infarct volume **(I)**, $n = 7$ (vehicle) and $n = 6$ (B4Crry), $*P < 0.05$ and $**P < 0.01$. **(J)** Kaplan-Meier curve representing 7-day survival of aged animals after MCAO. Mantel-Cox test, $n = 9$, $*P < 0.05$. **(K to N)** Experiments performed in adult male mice with 1-hour MCAO followed by reperfusion, with B4Crry administration 24 hours after ischemia. **(K)** Neurological deficit assessment over 15 days after MCAO, $n = 15$ (vehicle) and 18 (B4Crry). Two-way ANOVA with Bonferroni, $*P < 0.05$ and $**P < 0.01$. **(L)** Normalized laterality index on corner task. Multiple t tests with Holm-Sidak correction, $n = 9$ (vehicle) and $n = 9$ (B4Crry), $*P < 0.05$. **(M)** Cognitive performance on Barnes maze learning and retention 9 to 15 days after MCAO. Two-way ANOVA with Bonferroni, $n = 8$ (vehicle) and $n = 10$ (B4Crry), $**P < 0.01$. **(N)** Lesion volume reconstructed from Nissl-stained sections at 15 days after MCAO. Student's t test, $n = 10$, $***P < 0.001$. Three-dimensional models displayed with lesion in red. **(O)** Percentage of animals who died within 48 hours of MCAO because of intracerebral hemorrhage (ICH) after vehicle or B4Crry treatment. χ^2 test, $P > 0.05$, $n = 12$ per group. Data are reported as means $\pm$ SEM in all panels.

Fig. 4. B4Crry inhibits microglial phagoptosis of c-fos-expressing neurons acutely after ischemia. Experiments performed in adult WT male mice treated with B4Crry, B4scFv, or vehicle or in C3^{-/-} mice that underwent 1-hour MCAO followed by 24 hours of reperfusion. Treatments were administered 2 hours after ischemia (A) Top: Visualization of the location of selection of fields for IF staining for Figs. 4 and 5 using the mouse brain atlas. Bottom: IF for NeuN (red) showing neuronal density. (B) Quantification of neuronal cell density in three perilesional fields (240 μm × 240 μm) per animal (*n* = 5 animals per group). One-way ANOVA with Bonferroni, *n* = 5 per group, **P* < 0.05. (C) IF staining and super-resolution

microscopy of c-fos⁺ cells and microglia (Iba1⁺). **(D)** High-magnification volume reconstruction of fields from **(C)** using Amira showing c-fos material within microglial cell bodies with black arrows. Scale bars, 100 μ m. Fields are chosen using defined stereotactic location shown in **(A)**. **(E)** Quantification of neuronal c-fos⁺ cell density in three perilesional fields. One-way ANOVA with Bonferroni, $n = 5$ per group, $*P < 0.05$. **(F)** Quantification of cleaved caspase-3 expression in c-fos⁺ cells (IF staining shown in fig. S11). **(G)** Quantification of neuronal Iba1⁺ cell density in three perilesional fields. One-way ANOVA with Bonferroni, $n = 5$ per group, $*P < 0.05$ and $***P < 0.01$. **(H)** Frequency of c-fos material within microglial cell bodies. ANOVA with Bonferroni, $n = 4$, $***P < 0.001$. **(I and J)** High-resolution fields from the ipsilesional cortex showing c-fos and Iba1 localization (**I**) and Iba1 and NeuN localization (**J**). Left panels in **(D)** and **(E)**: Three-dimensional reconstruction of a 20- μ m stack using ZEN. Right panel in **(D)**: Orthographic view showing colocalization of c-fos (green) and Iba1 (red). Right panel in **(E)**: Orthographic view showing colocalization of Iba1 (green) and NeuN (red). Scale bars, 100 μ m. Insets in **(D)** show DAPI counterstain (in blue). **(K)** Quantification of the number of Iba1 cells containing c-fos material over total number of Iba1 cells. ANOVA with Bonferroni, $n = 4$ animals per group, $***P < 0.01$. **(L)** Quantification of the density of Mac2⁺ cells in the ipsilateral hemisphere. ANOVA with Bonferroni, $n = 4$ per group, $**P < 0.01$ and $***P < 0.001$. **(M)** Mac2 IF and localization of c-fos in perilesional brain areas. Scale bars, 100 μ m. **(N)** IF using anti-FCRLS antibody. Scale bars, 100 μ m. White boxes in **(D)** to **(F)** label regions of colocalization. **(O)** Quantification of IF shown in **(N)** and in fig. S11A showing percentage of c-fos⁺/Iba1⁺ clusters located within FCRLS⁺ cells. Data are reported as means $\pm$ SEM in all panels.

Fig. 5. Microglia phagocytosis of stressed neurons is complement C3d-dependent.

Experiments performed in adult male mice with 1-hour MCAO followed by 24 hours of reperfusion, with B4Crry or vehicle administered 2 hours after ischemia. (A) IF staining and super-resolution microscopy showing C3d, Iba1, c-fos, and DAPI in perilesional areas in vehicle-treated (A) or B4Crry-treated (B) mice. Left: Three-dimensional rendered view of 20-μm stack. Right: Orthographic view. Scale bars, 20 μm. For quantification of C3d deposition on c-fos⁺ neurons, refer to fig. S13. (C) Co-staining for NeuN, c-fos, Iba1, and C3d. Scale bars, 20 μm. Arrows point to site of C3d deposition at the microglia-neuron

interface. **(D)** Top: Schematic illustration of the approach used to quantify neuronal-microglial interaction. NeuN-Iba1 contacts were first identified and then classified on the basis of the presence of C3d or c-fos expression. Non-neuronal cells were not identified or classified. Bottom: Distribution of Iba1-NeuN contacts based on C3d and c-fos immunoreactivity. Fisher exact test was used to compare the proportion of C3d⁺ contacts between vehicle- and B4Crry-treatment groups, showing a significant difference ($P < 0.001$). **(E)** IF staining for c-fos material within microglia and localized to phagolysosomal vesicles (LAMP1⁺). Arrows and rectangle show the site of colocalization. **(F)** Representative 3D view of NeuN material localizing within microglial phagolysosomal vesicles (LAMP1⁺). White rectangle indicates region of colocalization. Scale bars, 20 μ m. **(G and H)** Quantification of LAMP1/c-fos/Iba1 overlay shown as intensity per pixel (G) and colocalized clusters (H). Black bars in (G) show the number of overlapping peaks. Student's *t* test, $n = 6$ animals (three fields per animal), $**P < 0.01$. Data are reported as means $\pm$ SEM in all panels.

Fig. 6. Acute complement activation initiates inflammatory cascades that persist chronically after stroke.

Experiments performed in adult male mice with 1-hour MCAO followed by reperfusion, with B4Crry or vehicle administered 2 hours after ischemia. (A to D) NanoString analysis of immunology-related gene expression 5 days after MCAO. (A) Clustergram of differentially expressed genes ($P < 0.01$, multiple t tests; see Supplementary Materials and Methods) between B4Crry- and vehicle-treated animals. Heat map represents fold expression relative to the average of the vehicle group for each gene. (B) Analysis of biological processes

enriched in the set of genes down-regulated by B4Crry. (C) Gene expression changes in complement genes 5 days after MCAO and B4Crry or vehicle treatment. $*P < 0.05$. Multiple t tests with Bonferroni, $n = 5$ per group, $P < 0.01$. (D) Gene expression changes in genes involved in microglia/macrophage attraction and activation. Red borders around bars indicate significance. Multiple t tests with Bonferroni, $n = 5$ per group. $P < 0.01$. (E and F) Representative serial images of IF staining of full-brain sections for Mac2⁺ and Ym1⁺ microglia (E) and quantification of Iba1 colocalization with Mac2 or YM1 (F). Scale bars, 200 μm . Student's t test. $n = 4$ animals (four fields per animal). $*P < 0.05$ and $**P < 0.01$. (G to I) Assessment of neurodegeneration by IF staining for neurons (NeuroTrace), dendrites (MAP2), and microglia (Iba1) 15 days after MCAO. (G) IF staining for neuronal (NeuroTrace), microglial (Iba1), and dendritic (MAP2) densities in the ipsilateral basal ganglia, cortex, and hippocampus in vehicle- and B4Crry-treated mice. Scale bar, 200 μm . Location of field selection and the approach used for quantification are shown in fig. S17. (H) Representative histograms from B54Crry- and vehicle-treated mice showing difference in dendritic density between the two groups. (I) Quantification of (G) and (H). Student's t test, $n = 5$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Data are reported as means $\pm$ SEM in all panels. FOV, field of view.

Fig. 7. Long-term complement deposition is associated with robust microgliosis and inflammatory cell infiltrate 15 days after MCAO.

Experiments performed in adult male mice with 1-hour MCAO followed by 15 days of reperfusion with B4Crry or vehicle administered 2 hours after ischemia (A) IF staining of the evolving infarct core showing Mac2, C3d, CD31, and DAPI expression. Insets 1 and 2: High-resolution fields from (A). (B) High-resolution fields from B4Crry-treated animals corresponding to same location as inset 1 in (A). Scale bars, 200 μm . (C) IF for C3d, CD31, and DAPI. Fields are 3D-rendered stacks covering 20 μm . Scale bars, 200 μm . White arrows

point to regions of overlap between C3d and CD31. (**D** and **E**) IF for CD3⁺ (**D**) and Gr1⁺ (**E**) cellular infiltrate in vehicle controls compared to B4Crry-treated animals. White bars, 200 μ m. Black bars, 50 μ m. (**F**) Quantification of (**D**) and (**E**). Student's *t* test, *n* = 5, **P* < 0.05 and ***P* < 0.01.