Targeting Mannose-Binding Lectin Confers Long-Lasting Protection With a Surprisingly Wide Therapeutic Window in Cerebral Ischemia

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Background—The involvement of the complement system in brain injury has been scarcely investigated. Here, we document the pivotal role of mannose-binding lectin (MBL), one of the recognition molecules of the lectin complement pathway, in brain ischemic injury.

Methods and Results—Focal cerebral ischemia was induced in mice (by permanent or transient middle cerebral artery occlusion) and rats (by 3-vessel occlusion). We first observed that MBL is deposited on ischemic vessels up to 48 hours after injury and that functional MBL/MBL-associated serine protease 2 complexes are increased. Next, we demonstrated that (1) MBL<sup>−/−</sup> mice are protected from both transient and permanent ischemic injury; (2) Polyman2, the newly synthesized mannosylated molecule selected for its binding to MBL, improves neurological deficits and infarct volume when given up to 24 hours after ischemia in mice; (3) anti-MBL-A antibody improves neurological deficits and infarct volume when given up to 18 hours after ischemia, as assessed after 28 days in rats.

Conclusions—Our data show an important role for MBL in the pathogenesis of brain ischemic injury and provide a strong support to the concept that MBL inhibition may be a relevant therapeutic target in humans, one with a wide therapeutic window of application. (Circulation. 2012;126:1484-1494.)

Key Words: cerebral ischemia ■ endothelium ■ inflammation ■ stroke ■ complement system

Stroke is a leading cause of death and permanent disability worldwide. Despite recent substantial progress in prevention and management, stroke still remains a large unmet medical need. Ischemic stroke, that includes 87% of all strokes and is caused by thrombotic or embolic occlusions, occurs when the blood supply to part of the brain is restricted, leading to energy deficit. In areas with the lowest residual flow, cells die rapidly, whereas in areas with less severe ischemia that surround the lesion core, cells may remain viable for hours to days and represent possible therapeutic targets. Presently, the only treatment available for stroke is thrombolysis with tissue plasminogen activator. The narrow 3-hour therapeutic window imposed by safety concerns (only recently extended to 4.5 hours<sup>2</sup>) allows <5% of patients to be eligible for tissue plasminogen activator treatment.<sup>3,4</sup> Thus, the inadequacy of available therapeutic strategies demands the identification of novel ameliorative treatments endowed with a wide therapeutic window.

Clinical Perspective on p 1494

Several pathogenetic mechanisms contribute to ischemic injury. Among them, the inflammatory response, after being rapidly triggered, progresses over hours to days, making it an attractive target for therapeutic intervention. The complement system is a powerful arm of inflammation. It becomes rapidly activated after injury and acts as a trigger for several aspects of the inflammatory response. Complement is a proteolytic cascade of several circulating and cell-associated proteins that contribute to the evolution of injury by several potential
mechanisms, including inflammatory molecule synthesis, recruitment of cells to the site of injury, activation of phagocytosis, induction of endothelial damage and increased vascular permeability, and by directly inducing cell death. Depending on the trigger, complement activation may proceed through 3 separate pathways, namely, the classical, lectin, and alternative pathways. Both animal models and clinical observations indicate that the complement system is one of the mechanisms contributing to ischemic injury.6–8

C1-INH, an endogenous inhibitor of the complement system endowed with several anti-inflammatory effects, acts as a potent neuroprotective agent against acute brain injury.9–13 We showed previously that recombinant C1-INH markedly reduced cerebral damage when administered up to 18 hours after ischemia in mice.12 Our data further suggested that the remarkable protective action was due to its binding to mannose-binding lectin (MBL), one of the recognition molecules responsible for the first step of complement activation of the lectin pathway.14 MBL is a serum protein that acts as a circulating pattern recognition receptor. MBL targets include pathogen-associated molecular patterns, ie, carbohydrates exposed on the surface of a pathogen but also “altered self” or damage-associated molecular patterns, ie, dying or damaged cells through recognition of changes in glycosylation pattern on the cell surface. The binding of MBL to its targets leads to complement activation.14

Our previous data prompted us to hypothesize that MBL plays a pathogenetic role in the ischemic injury and that compounds that bind and inhibit MBL may represent a promising therapeutic modality for stroke. Here, we documented the pivotal role of MBL in cerebral ischemic injury by using different experimental models of focal ischemia. Because the degree of reperfusion is variable in the clinical setting,1,15 we used both transient and permanent middle cerebral artery occlusion (tMCAo and pMCAo, respectively) in mice.9,10,12 In addition, an inhibitory monoclonal antibody against rat MBL-A16 was also used in a reversible 3-vessel occlusion model (3-vo) in rats.17

Methods

Animals

Male C57Bl/6 (26–28 g, Harlan, Italy, and Taconic Farms for the complement hemolytic assay) and C57Bl/6 with target mutation of both MBL-A and MBL-C genes (MBL−/−, 26–28 g, Jackson Laboratories, mice, and Crl:CD (SD)BR (250–280 g, 7–8 weeks, Charles River, Calco, Italy) rats were used. Additional information in online-only Data Supplement Methods.

Drugs

Anti-rat MBL-A monoclonal antibodies (clones P2D5 and 14C, 1 mg/kg) were diluted in phosphate-buffered saline and injected intravenously.16,18 Polyman2 was dissolved in phosphate-buffered saline at a concentration of 450 (or 900) μM. One hundred microliters of this solution were injected intravenously to obtain circulating levels corresponding to 30 (or 60) μM, corresponding to 142 (or 284) μg per mouse, which represented the best binding concentration to MBL in surface plasmon resonance (SPR) experiments. Additional information on Polyman2 toxicity is in online-only Data Supplement Methods.

Focal Ischemia in Mice: tMCAo and pMCAo

Surgery

Transient ischemia was achieved by tMCAo by means of a siliconeized filament (7-0, Doccol Corporation) introduced into the internal carotid artery and advanced to block the MCA for 30 minutes as described previously.9,10,19 The surgery-associated mortality rate was 8%. For permanent ischemia (pMCAo), the MCA was permanently occluded by electrocoagulation.20,21 The mortality rate for this model was 8.5%. In both ischemia models, sham-operated (sham) mice received identical anesthesia and surgery without artery occlusion.

Neurological Deficits

Forty-eight hours after tMCAo, each mouse was rated on neurological function scales unique to the mouse. Scores range from 0 (healthy) to 56 (the worst performance in all categories) and represent the sum of the results of general and focal deficits (13 categories). Results are expressed as a composite neurological score.

Quantification of Infarct Size and Edema

Infarct volumes were calculated on 20-μm coronal brain cryosections stained with cresyl violet by the integration of infarcted areas after correction for the percentage of brain swelling due to edema. Edema was determined by subtracting the area of the ipsilateral from that of the contralateral hemisphere.

Additional information on surgery, neurological deficits, and quantification of infarct size and edema is in online-only Data Supplement Methods.

Focal Ischemia in Rats: 3-vo

Surgery

Surgery was performed as described previously.17,22 The right common carotid artery and the MCA were occluded. Subsequently, to produce a lesion in the MCA region, the contralateral common carotid artery was occluded for 1 hour by the use of traction with fine forceps. This procedure induces a blood flow drop in the MCA territory. Reperfusion was induced by forceps release. Sham rats were operated on in the same way, but the MCA and common carotid arteries were not occluded. The surgery-associated mortality rate was 7%.

Neurological Deficits

Postural reflex was assessed by the Bederson test, sensorimotor integration by the De Ryck limb-placing test, and the ability to integrate motor responses by the foot-fault test as previously described.17

Quantification of Infarct Size

Injury was quantified on 14 serial 1-mm thick sections stained with triphenyltetrazolium chloride (Sigma-Aldrich).17 Alternatively, 4 weeks after ischemia, ischemic volume was evaluated by structural MRI analysis (see below).

Additional information on surgery, neurological deficits, and quantification of infarct size is in online-only Data Supplement Methods.

MRI Measurements

Imaging was performed on a 7T small-bore animal Scanner (Bruker Biospec). The morphological images were obtained with a RARE T2-weighted sequence that covered the whole rat brain volume.

Volumetric Measurements

The volume measurements of structural MRI images were obtained manually by using custom-made software as previously described.23 For each animal, the regions of interest were manually chosen and drawn on the images for volumetric assessments. We computed the lesion volume including the whole infarcted tissue (T2w hypertensive tissue indicative of edema) across different brain areas. This measurement did not include the ventricle volume. Measurements of brain atrophy in cortex, hippocampus, and striatum included residual T2w hypointense tissue. Data from each animal were obtained by the integration of averaged region of interest area for
slice thickness. Additional information is in online-only Data Supplement Methods.

Immunofluorescence and Confocal Analysis
Twenty-micrometer-thick coronal sections were incubated with the primary monoclonal antibodies anti-mouse MBL-A or MBL-C (1:100; Hycult Biotechnology). Alexa546 fluorescent-conjugated goat–anti-rat IgG (1:500; Molecular Probes) was used as a secondary antibody. Brain vessels and nuclei were stained with Alexa488 fluorescent-conjugated isothiocyanate (IB4; 1:200; In vitrogen) and 4’-6-diamidino-2-phenylindole (1 µg/mL; Invitrogen), respectively. Images were acquired by confocal microscopy as described previously. Three-dimensional images were acquired over a 10- to 12-µm z axis with a 0.23-µm step size and processed by using Imaris software (Bitplane) and Photoshop CS2 (Adobe Systems Europe Ltd).

Semiquantitative evaluation of MBL deposition on blood vessels was performed at each time point. Additional information is in online-only Data Supplement Methods.

Functional MBL/MBL-Associated Serine Protease 2 Assay
Blood samples were collected from the vena cava in 10 mmol/L EDTA and 0.125% polybrene (Sigma Aldrich). Functional MBL/MBL-associated serine protease 2 (MASP-2) complexes were measured in plasma by ELISA (Hycult Biotechnology).

Western Blot
Equal amounts of plasma proteins (10 µg per sample) were electrophoresed on a 5% sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene fluoride membranes. Rabbit anti-C3 polyclonal antibody (1:100) followed by rabbit peroxidase-conjugated antibody (1:2500; both Santa Cruz Biotechnology) were used. Results were standardized by using the total protein loaded. Additional information is in online-only Data Supplement Methods.

Surface Plasmon Resonance
SPR binding studies were performed by using the ProteOn XPR36 system (Bio-Rad), as previously described. In brief, polycyanosylated dendrimers were flowed onto recombinant human MBL covalently immobilized on the sensor chip. The resulting sensograms were fitted by the simplest 1:1 interaction model to obtain the binding constants. Additional information is in online-only Data Supplement Methods.

Complement Hemolytic Assay
Mice were divided in the following groups (n=3/group): (1) wild type+saline (vehicle control) and (2) wild type+Polyman2. Under isoflurane anesthesia, 50 µL of normal saline (control) or 50 µL of saline containing 142 µg of Polyman2 was injected intravenously. After 1 hour, blood was collected via heparinized syringes and serum 13/86 and rats: 2/77).

Blinding and Exclusion Criteria
In each and every experiment, animals (including C57Bl/6 and MBL−/− mice and Crl:CD(SD)BR rats) were allocated at random to surgery and treatment group, taking care to distribute them equally across experimental days and batches to avoid systematic errors. All the experimental procedures including surgery, drug treatment, behavioral tests, immunohistochemistry, quantification of infarct size, MRI analysis, and biochemical assays were performed by investigators blinded to the experimental conditions. Animals, in which the intravenous administration of drug or vehicle was not successful, were excluded from the study (mice: 13/86 and rats: 2/77).

Statistics
Data are expressed as mean±SD or as scatter-dot plots and mean (bars). GraphPad Prism 5 software was used for statistical analysis. All of the data have been checked for normal distribution by the Kolmogorov-Smirnov test and for the constancy of variances by the Bartlett test, in case of >2 groups, or by F test, in case of 2 groups. The Kruskal-Wallis test was used when data were not distributed normally. The t test with the Welch correction was used for normally distributed data with unequal variances. Additional information on the tests used are reported in the figure legends and in online-only Data Supplement Methods.

Results
The Lectin Pathway in Brain Ischemia
We first investigated whether MBL could be detected in the ischemic brain. We assessed the presence of MBL-A and MBL-C, the 2 isoforms present in mice,27 at 30 minutes and at 6, 12, 24, and 48 hours following tMCAo or pMCAo (Figure 1). MBL was detected in tMCAo mice starting from 6 hours and in pMCAo mice from 30 minutes. In both models, the strongest signal was observed at 12 to 24 hours and persisted up to 48 hours after ischemia (Figure 1E and 1F). Both MBL-A and MBL-C were present on ipsilateral vessels of ischemic mice and deposited on the luminal side of the vessels after tMCAo (Figure 1A and 1C) or pMCAo (Figure 1B and 1D), as evidenced by single-plane (Figure 1A’ through 1D’) and 3-dimensional images (Figure 1A” through 1D”). Coronal sections of vessels were obtained by clipping the 3-dimensional renderings (Figure 1A” through 1D”) and further confirmed the intraluminal deposition of both MBL isoforms. Sham-operated mice showed only occasional, transient deposition of MBL 30 minutes after sham injury (online-only Data Supplement Figure I). No MBL signal was detected at longer time points in sham mice or in the contralateral side of the ischemic animals (data not shown).

We next assessed the presence of circulating MBL/MASP-2 functional complexes, as an index of lectin pathway activation. We previously demonstrated that tMCAo induced a significant increase in MBL/MASP-2 complexes in comparison with sham-operated mice.12 In the present study, we found that both 30 minutes and 24 hours after pMCAo, ischemic mice had a significant increase (up to 26.6% and 31.9%, respectively) in circulating functional MBL/MASP-2 complexes in comparison with sham-operated mice (Figure 2A and 2B), indicating an early and persistent activation of the lectin complement pathway following pMCAo. Notably, 24 hours after pMCAo, ischemic mice had a significant increase in circulating C3 fragments in comparison with sham-operated mice (up to 64.5%; Figure 2C), indicating a long-lasting activation of the complement system.

Susceptibility of MBL−/− Mice to MCAo Injury
To further establish the relevance of MBL after ischemic injury, we then assessed the susceptibility of MBL−/− mice to ischemia. First, we assessed cerebral blood flow perfusion rates by laser Doppler flowmetry (Perimed Italy) and found that in MBL−/− they were not different from wild-type mice (online-only Data Supplement Figure II). Similarly to what was observed after tMCAo (online-only...
MBL indicates mannose-binding lectin; tMCAo, transient middle cerebral artery occlusion; and pMCAo, permanent middle cerebral artery occlusion.

**Figure 1.** MBL is deposited on ischemic vessels after tMCAo and pMCAo. Representative images of staining for MBL-A or MBL-C (red), vessels (IB4, green), and nuclei (Hoechst, blue) in the ipsilateral cortex 12 hours after tMCAo (A and C) or pMCAo (B and D). After tMCAo, both MBL-A (A) and MBL-C (C) show deposition on ischemic vessels. Single $xy$ plane views with $z$ projections of the white boxes in A and C confirm that both isoforms were located in the luminal space of vessels (A' and C'). Three-dimensional renderings (A' and C') and clipped volumes (A'' and C''); clipping in correspondence of the yellow planes) further demonstrate intraluminal deposition of MBL-A and MBL-C. After pMCAo, MBL-A and MBL-C show similar intraluminal deposition by fluorescence (B, B', D, and D'), 3D renderings (B' and D') and clipped volumes (B'' and D''). Confocal analysis, scale bar=20 μm (A through D) and 5 μm (A' and D').

## Data Supplement Figure II

28, 29 MBL $^{-/-}$ mice subjected to pMCAo had smaller lesions in comparison with wild-type mice, corresponding to a 48% decrease of infarct volume (Figure 2D).

## SPR Analysis of Mannosylated Molecules

We then sought to identify a synthetic MBL ligand directed against the mannose recognition binding site (carbohydrate recognition domain, CRD) to be used as a possible...
therapeutic tool in ischemic injury. Different polymannosylated dendrimers, including Polyman1 and Polyman2,\textsuperscript{30} and the new molecules Polyman6, Polyman5, Polyman8, Polyman17, Polyman12, and Polyman14 (R. Ribeiro, N. Varga, I. Sutkevičiute, F. Fieschi, A. Bernardi, J. Rojo, 2012, unpublished data), were compared for their ability to bind MBL by SPR assay (Figure 3A and online-only Data Supplement Figure III). These studies highlighted the importance of both the number of pseudomannosides present on the dendrimeric scaffold (online-only Data Supplement Figure 3) and the structure of the scaffold (eg, Polyman2 versus Polyman17 in Figure 3A), and indicated Polyman2 as the best MBL ligand, with an affinity of 6 μmol/L (Figure 3B).

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**Figure 2.** The lectin pathway is involved in pMCAo injury. Functional MBL/MAST-2 complexes in mice plasma samples collected 30 minutes (A) or 24 hours (B) after sham surgery or pMCAo. (C) C3 complement activation fragments in plasma samples collected 24 hours after sham surgery or pMCAo. (D) Infarct volume assessed 48 hours after pMCAo in WT and MBL/H\textsuperscript{-/-} mice. Data are reported as scatter-dot plots and mean (bars), n=8 to 10 mice per group, unpaired t test; *P<0.05, **P<0.01, ***P<0.001. MBL indicates mannose-binding lectin; MASP-2, MBL-associated serine protease 2; pMCAo, permanent middle cerebral artery occlusion; and WT, wild type.

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**Figure 3.** Binding of mannosylated dendrimers to MBL evaluated by SPR. MBL was immobilized on the surface of sensor chip, whereas the dendrimers were injected for 3 minutes at a flow rate of 100 μL/min. A, the maximal binding (in resonance units, RU) obtained when injecting 100 μmol/L dendrimers with different structures. The data shown are from a single experimental session, but the same rank order was obtained in independent injections, with the use of different concentrations or different flow rates. No binding was observed on a parallel surface immobilizing BSA. B, the sensorgrams obtained by injecting 4 different concentrations of Polyman2 (black lines), together with the corresponding fittings (white lines). The analysis of these sensorgrams allowed estimation of the binding constants shown. MBL indicates mannose-binding lectin; SPR, surface plasmon resonance; and BSA, bovine serum albumin.

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A

| Tetra-valent Mono-mannoside | Polyman5 |
| Tetra-valent Di-mannoside | Polyman8 |
| Tetra-valent Tri-mannoside | Polyman17 |
| Tetra-valent Di-mannoside | Polyman1 |
| Tetra-valent Tri-mannoside | Polyman2 |

**binding (RU) at 100 uM**
Effect of the Mannosylated Molecule Polyman2 in MCAo Injury

Polyman2 was therefore selected for the in vivo studies and administered intravenously to tMCAo mice, at doses that reached plasma concentrations of 30 or 60 μmol/L (142 or 284 μg per mouse, respectively) to mimic the concentrations that were functionally active in SPR experiments. Both doses, given at reperfusion (30 minutes from the beginning of ischemia), induced a marked reduction in ischemic volume by 33.2% and 34.2% in comparison with vehicle-treated mice (online-only Data Supplement Figure IV). Thus, all subsequent experiments were performed by using 30 μmol/L Polyman2.

First, we demonstrated that Polyman2 did not induce complement depletion in vivo. Plasma from vehicle- or Polyman2-treated mice restored C5 activity to human C5-depleted sera to the same extent over a wide range of plasma concentrations (Figure 4A). Next, to explore the therapeutic window against ischemic injury, Polyman2 or vehicle was infused intravenously once at different time points following tMCAo (3, 6, 12, 18, 24, or 30 hours from the beginning of ischemia). Neurological deficits, determined 48 hours after tMCAo, indicated that Polyman2 significantly reduced ischemic functional deficits when given up to 30 hours after the ischemic insult in comparison with vehicle-treated mice (Figure 4B) and reduced the ischemic volume by 46.4% when given 24 hours after ischemia (Figure 4C). Notably, in the absence of any treatment, the ischemic lesion increases by 55% from 24 to 48 hours (36.8±13.2 versus 56.2±16.2, n = 12). The strongest protective effect (68.3% reduction) was observed when Polyman2 was given 6 hours following tMCAo (Figure 4C). At this time point, the maximal effect of Polyman2 on reduction of brain edema was also observed (online-only Data Supplement Figure V).

In Polyman2-treated mice, the ischemia-induced MBL deposition on brain endothelium was effectively dampened at 24 hours (Figure 4D), and circulating C3 fragments were decreased 48 hours after injury (Figure 4E and 4F). Importantly, Polyman2 given 6 hours after injury to MBL−/− mice was not able to affect either the functional or the anatomic damage, showing the specificity of the protective effect (Figure 4G and 4H).

Mice receiving Polyman2 treatment 3 or 6 hours after pMCAo did not show a significant decrease of the lesion size nor reduction of MBL deposition or of C3 fragmentation (online-only Data Supplement Figure VI).

Effect of the Anti-MBL-A Antibody Administration

Because no functionally inhibitory antibody against mouse MBL is available, we then assessed the effect of an anti-MBL monovalent antibody directed against rat MBL-A (mAb P2D5 clone16), the most abundant MBL isoform in rats.27 P2D5 has potent neutralizing properties against MBL-A but does not recognize MBL-C.16 This clone (1 mg/kg16) or the vehicle was administered intravenously 20 minutes before ischemia or 6, 18, or 24 hours in rats subjected to reversible 3-vo.22 Neurological deficits and ischemic volume were determined 48 hours after injury. Both postural-reflex deficits (the Bederson test; Figure 5A) and sensorimotor deficits (the De Ryck test; Figure 5B) were significantly improved in all mAb-treated rats, even those receiving the antibody 18 hours after injury. The ability to integrate motor responses (foot-fault test; Figure 5C) was also improved in rats treated up to 18 hours after injury, but it did not reach statistical significance. Evaluation of anatomic injury demonstrated that rats treated with the mAb up to 18 hours after ischemia had a significant reduction in ischemic volume (posttreatment at 18 hours: 19% reduction; Figure 5D).

We also assessed the specificity of the protective effect elicited by P2D5 by administering mAb 14C that binds rat MBL-A but is not functionally inhibitory18 (isotype control antibody; Figure 5E). When injected 18 hours after 3-vo, mAb 14C did not ameliorate any of the functional deficits or anatomic damage in comparison with vehicle-treated rats (Figure 5E).

In a subsequent experiment, rats were treated with P2D5 (1 mg/kg) 18 hours after ischemia and were analyzed serially out to 28 days to assess whether the antibody’s protective effect was long lasting. Postural and sensorimotor deficits and the ability to integrate motor responses were significantly improved in mAb-treated rats up to 28 days (Figure 6A through 6C). Because it is not practical to assess classical histological injury at this late time point, we analyzed the ischemic rat brains by MRI, evaluating the extension of the lesion and tissue atrophy. We found that antibody-treated rats had a significant reduction in the overall ischemic volume (58.2%; Figure 6E), and less atrophy in the hippocampus (Figure 6F) and cortex (Figure 6G), as well, but not in striatum (not shown) in comparison with vehicle-treated rats.

Discussion

This study establishes a pivotal role for MBL in the pathogenesis of brain ischemic injury and demonstrates in multiple models and with the use of 2 structurally different inhibitors that MBL’s inhibition leads to protection with a surprisingly wide therapeutic window. We have shown that MBL is deposited on the ischemic endothelium after injury (with and without reperfusion) and that functional MBL/MASP-2 complexes are increased following pMCAo similarly to what is observed after tMCAo,13 indicating lectin pathway activation by the ischemic injury. Furthermore, by using 3 independent lines of evidence, we demonstrated that MBL inhibition is protective by observing the following: (1) the susceptibility of MBL−/− mice to tMCAo and pMCAo is decreased in comparison with wild-type mice; (2) Polyman2, a new dendrimeric molecule binding MBL with high affinity, induces a significant reduction of neurological deficits and ischemic volume when given up to 24 hours after injury induction; and (3) the anti-MBL mAb induces a significant and long-lasting reduction of neurological deficits and ischemic volume when given up to 18 hours after the induction of ischemic injury in rats.

The most relevant finding of this study is the surprisingly wide therapeutic window that can be obtained by administering either Polyman2 or the anti-MBL mAb. Interestingly, in 2 different models of ischemia/reperfusion injury (tMCAo in
mice and 3-vo in rats), the extent of the protection and the effective therapeutic window were very similar. We do not know if the deposition of MBL on the ischemic vessels has a direct pathogenetic effect, but the observation that it decreases in tMCAo mice treated with Polyman2 (and not in pMCAo mice in which Polyman2 is not effective) suggests that this may be the case. Thus, the intraluminal deposition of MBL may be the important event implying that the drugs (Polyman2 or the antibody) do not need to cross the blood brain barrier to be effective.

Notably, MBL deposition is triggered early after ischemia and lasts up to 48 hours. Thus, we hypothesize that the wide therapeutic window may be a result of targeting such a long-lasting event.

Recent data implicate MBL and/or the lectin pathway in reperfusion injury in several organs.31–36 Here, we demon-
strate that MBL-/- mice are protected even in the absence of reperfusion. MBL-/- mice subjected to pMCAo show a dramatic reduction of the lesion volume in comparison with wild-type mice. Similar to observations in tMCAo (present results and references 28, 29), pMCAo mice deposit MBL on ischemic vessels, increase levels of functional MBL/MASP-2 complexes, and increase circulating C3 fragments, thus implicating that activation of the lectin pathway following brain ischemia does not need reperfusion. However, Polyman2 was ineffective in reducing the ischemic lesion when administered to pMCAo mice, possibly because of its inability to reach the vessels in the ischemic area owing to the permanent vessel occlusion. Alternatively, we cannot exclude that higher doses could be needed for Polyman2 to reach the areas where MBL is deposited on the ischemic endothelium following pMCAo. However, because, in the clinical setting, areas lacking reperfusion are mingled with reperfused areas,1,15 this limitation would not, in principle, prevent a possible efficacy in human stroke.

MBL circulates in complexes with serine proteases (MASPs) and other nonenzymatic proteins.37 On MBL binding to its carbohydrate target, MASPs are activated and cleave C4 and C2 to form the C3 convertase, which may lead to activation of the entire complement system (eg, through to formation of C5b-9). Circulating MBL recognizes highly mannosylated glycoproteins expressed on the surface of pathogens or of altered self-cells through its C-terminal CRD.14,38 Functional assembly into oligomeric structures allows formation of multiple CRD domains (clusters of 2–6 CRDs), which leads to an efficient binding complex with multivalent properties. Although the binding affinity of each individual interaction between the CRD and the mannosylated glycoproteins is relatively low, the self-assembly into high-order oligomers provides a way for MBL to recognize repetitive arrays of its carbohydrate targets with high avidity. Thus, to effectively interfere with these multivalent interactions, multivalent inhibitors have to be designed. Polyman2 (previously referred to as Dendron 1230) is a tetravalent pseudotrimannoside dendron. It is formed by a tetrivalent polyester scaffold, decorated with a mimic of linear 1,2- to 1,6-trimannoside. Polyman2 was selected as the most active MBL-binding complex by SPR analysis from a small group of congeners that varied in structure of the dendron scaffold and the nature of the mannose-based ligand. Polyman2 has a high solubility in physiological media, negligible cytotoxicity, and a stability of 6 to 12 hours at pH 7.4 in aqueous solution.30 In vivo, on administration to ~60 ischemic...
mice we could not detect any apparent sign of toxicity. Importantly, Polyman2 does not activate and deplete complement.

Because of the presence in vivo of several structures potentially capable of recognizing mannosides, Polyman2 could also act on other CRD targets. The inability to exert an additional protective action in MBL mice suggests that the protective effect of Polyman2 involves its interaction with MBL.

MBL is widely known as the first step of activation of the lectin complement pathway. We cannot exclude that MBL may have a direct toxic effect, independent from its ability to activate complement. Recent data obtained in a renal ischemia injury model show that MBL-mediated tubular injury is independent of complement activation and that MBL-mediated cell death precedes complement activation. Thus, future studies are needed to fully elucidate this aspect in brain injury.

In humans, MBL is encoded by a single gene, whereas in rodents it is encoded by 2 different genes, MBL-A and MBL-C. Both rodent MBLs form higher oligomeric structures and activate complement. They display slightly different ligand specificities and may be differently expressed in different tissues; however, whether they also possess other common or divergent biological functions is not known yet.

Figure 6. Anti-MBL-A antibody protection form ischemic injury is long lasting. Anti-MBL-A mAb (P2D5 clone, 1 mg/kg IV) was given 18 hours after 3-vo (at arrow). Postural-reflex deficit (A, Bederson test; normal score=5), sensorimotor deficit (B, De Ryck test; normal score=2), were assessed 17 hours (pretreatment time), 2, 7, 14, and 28 days after 3-vo in vehicle- or anti-MBL-A antibody-treated rats or in sham-operated rats. Data are reported as mean±SD, n=7 per group. Two-way ANOVA for repeated measures followed by Bonferroni post hoc test (A through C): *P<0.05, **P<0.01, ***P<0.001, Ab P2D5 versus vehicle-treated rats at each given time. Structural MRI data analysis of brain volumes (T2w) in ischemic rats treated with vehicle or with anti-MBL-A antibody: total lesion volume (E, measured as the whole T2w hyperintense tissue across brain areas, red ROI in D) and atrophy (measured as residual T2w hypointense tissue) in hippocampus (F, yellow ROI in D) and cortex (G, green ROI in D) for ipsi- and contralateral areas. Asterisks (blue) identify ventricles. Data are reported as scatter-dot plots and mean (bar), n=7 per group. Two-way ANOVA for matched values (ipsi- and contralateral sides) followed by Bonferroni post hoc test (F and G) and unpaired t test with a Welch correction (E): *P<0.05, **P<0.01, ***P<0.001, comparisons as indicated by horizontal lines. Ab indicates antibody; MBL, mannose-binding lectin; 3-vo, 3-vessel occlusion model; and ROI, region of interest.
Detailed analysis of the MBL gene in humans has revealed that a surprisingly high percentage of individuals (15%–30% depending on the population considered) carries a genetic deficiency in MBL that leads to low circulating levels of serum MBL.42 These subjects have not been found to be prone to severe infections in prospective studies; rather, the high diffusion of MBL-low individuals suggests that a relative lack of MBL may be beneficial to the host under certain circumstances.43 Notably, 2 human studies have recently investigated the role of MBL in stroke and found that a genetic deficiency in MBL is associated with a better outcome after acute stroke in humans,28,44 thus further strengthening the hypothesis that inhibition of MBL in the clinical setting of stroke may have significant and beneficial outcomes.

As recently highlighted,45 MBL has been, up to now, a somewhat forgotten molecule. However, recent data are strongly suggesting an important role for MBL and the lectin pathway in the pathogenesis of brain injury.12,28,44,46 Our data provide strong support to the concept that MBL inhibition may be a relevant therapeutic target in humans, one with a wide therapeutic window of application. Thus, we propose MBL as a novel therapeutic target for stroke.

Acknowledgments

The authors thank Carlotta Ceriani and Daiana De Blasio for skillful technical assistance and Mauro Tettamanti for statistical advice.

Sources of Funding

This study was supported in part by Italian Ministry of Health, Young Investigators Award 2009 (to Dr Zanier), and by Cariplo Funded by National Institutes of Health grants AI089781, HL056086, Young Investigators Award 2009 (to Dr Zanier), and by Cariplo Foundation.

Disclosures

None.

References

Despite recent substantial progress in prevention and supportive care, stroke is still a leading cause of death and permanent disability worldwide. To date, thrombolysis with tissue plasminogen activator is the only available treatment and its narrow therapeutic window (3–4.5 hours) is one of the main obstacles to finding eligible patients. Thus, new approaches with a wider window of efficacy are needed. This study documents the pivotal role of mannose-binding lectin (MBL), a circulating protein that acts as the first step in activation of the lectin complement pathway in brain ischemic injury. The data show that MBL deposition on the ischemic endothelium represents a key pathogenetic event in brain damage. Importantly, strategies aimed at inhibiting MBL lead to neuroprotection with a time window of efficacy up to 24 to 30 hours postinjury, an extremely important factor in the attempt to translate experimental results into the clinical setting. Detailed analysis of the MBL gene in humans has revealed that a surprisingly high percentage of individuals (15%–30% depending on the population considered) carries a genetic deficiency in MBL that leads to low circulating levels of MBL. Notably, this deficiency is associated with a better outcome after acute stroke in humans. Our data, providing a mechanistic insight into the role of MBL in brain ischemia and the demonstration that its inhibition is protective, strongly support the concept of MBL as a relevant therapeutic target in humans, one with a wide therapeutic window of application. Thus, we propose MBL as a novel therapeutic target for stroke.

**CLINICAL PERSPECTIVE**

Targeting Mannose-Binding Lectin Confers Long-Lasting Protection With a Surprisingly Wide Therapeutic Window in Cerebral Ischemia
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Circulation. 2012;126:1484-1494; originally published online August 9, 2012; doi: 10.1161/CIRCULATIONAHA.112.103051
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/126/12/1484

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2012/08/09/CIRCULATIONAHA.112.103051.DC1

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SUPPLEMENTAL MATERIAL

Supplemental methods

Animals


Drugs

To test Polyman2 toxicity single-dose toxicity tests, in conformity with OECD Guideline for Testing of Chemicals, and Directive 75/318/EEC EEC, were conducted on male mice. No toxicity was apparent in mice receiving Polyman2 30 or 60 µM intravenously when observed up to two months after drug administration (not shown).
Focal ischemia in mice: transient and permanent middle cerebral artery occlusion.

**Surgery.** Anesthesia was induced in mice by 3% isoflurane in a N\textsubscript{2}O/O\textsubscript{2} (70/30%) mixture and maintained by 1-1.5% isoflurane in N\textsubscript{2}O/O\textsubscript{2} (70/30%) mixture. Transient ischemia was achieved by middle cerebral artery occlusion (MCAo)\textsuperscript{1-3} by means of a siliconized filament (7-0, Doccol Corp.) introduced into the internal carotid artery and advanced to block the MCA for 30 min. At the end of the ischemic period the filament was withdrawn and reperfusion allowed. Surgery-associated mortality rate was 8%. For pMCAo mice were anesthetized with Equitensin (pentobarbital 39 mM, chloral hydrate 256 mM, MgSO\textsubscript{4} 86 mM, ethanol 10% v/v, propyleneglycol 39.6% v/v) 100 µl/mouse administered by intraperitoneal injection. The right MCA was exposed through a small burr hole in the left temporal bone and permanently occluded by electrocoagulation \textsuperscript{4, 5}. Mortality rate for this model was 8.5%. In both ischemia models, sham-operated (sham) mice received identical anesthesia and surgery without artery occlusion. All mice were maintained at 37°C during surgery using a heating pad (LSI Letica).

**Neurological deficits.** Forty-eight hours after tMCAo each mouse was rated on neurologic function scales unique to the mouse. Scores range from 0 (healthy) to 56 (the worst performance in all categories) and represent the sum of the results of all categories. These include general deficits: hair (0–2), ears (0–2), eyes (0–
4), posture (0–4), spontaneous activity (0–4), and epileptic behavior (0–12), and focal deficits: body symmetry (0–4), gait (0–4), climbing on a surface held at 45° (0–4), circling behavior (0–4), front limb symmetry (0–4), compulsory circling (0–4), and whisker response to a light touch (0–4). Results are expressed as composite neurological score.

Quantification of infarct size and edema. Perfused brains were obtained as described previously. Twenty-micron coronal brain cryosections were cut serially at 320 µm intervals and stained with cresyl violet (Sigma-Aldrich). On each slice, the infarcted area was assessed blindly and delineated by the relative paleness of histological staining tracing the area on a video screen. Infarct volumes were calculated by the integration of infarcted areas after correction for the percentage of brain swelling due to edema. Edema was determined by subtracting the area of the ipsilateral from that of the contralateral hemisphere. Quantifications were performed using a computer-assisted image analyzer and calculated by Analytical Image System (Imaging Research Inc.)

Focal ischemia in rats: three vessel occlusion (3-vo).

Surgery. Rats were anesthetized with intraperitoneal chloral hydrate (400 mg/kg) and surgery was done as described previously. Briefly, the common carotid arteries (CCAs) were visualized and the right one occluded. A hole adjacent and rostral to the right orbit allowed visualization of the MCA, which was cauterized distal to the rhinal artery. To produce a lesion in the MCA region, the contralateral CCA was occluded for 1h using traction with fine forceps. This procedure induces a blood flow drop in the MCA territory. Reperfusion was induced by forceps.
release. Sham rats were operated the same way but the MCA and CCAs were not occluded. Surgery-associated mortality rate was 7%.

**Neurological deficits.** In the postural reflex test of Bederson, rats were scored as follows: grade 5, normal; grade 4, moderate (forelimb flexion and no other abnormalities); grade 3, severe (reduced resistance to lateral push toward the paretic side, and forelimb flexion); grade 2, severe (same behavior as grade 3, with circling toward the paretic side when pulling the tail on the table); grade 1, severe (same behavior as grade 2, with spontaneous circling); grade 0, no activity. De Ryck’s limb-placing test examines sensorimotor integration in limb placing responses to visual, vibrissae, tactile, and proprioceptive stimuli. For each test, limb placing scores were 0, no placing; 1 incomplete and/or delayed (>2 s); or 2, immediate and complete placing. Each test was repeated for each paw up to 10 times and for each body side the maximum limb placing score was 16. The foot fault test measures the animal’s ability to integrate motor responses. The rats were placed on a grid with 2 cm spaces between 0.3 cm diameter metal rods and were observed for 2 min. With each weight-bearing step, the paw can fall or slip between the wires and this was recorded as a foot fault. The number of foot faults for the paws contralateral and ipsilateral to the infarction was recorded with the number of successful steps, and the foot fault index was calculated as the percentage of contralateral limb foot faults per limb step minus the percentage of ipsilateral limb foot faults per limb step.

**Quantification of infarct size.** For infarct size assessment, the brains were removed, transferred to cold saline and 14 serial 1-mm thick sections were cut
through the entire brain. Then sections were stained with a solution of 1% (w/v) triphenyltetrazolium chloride (TTC, Sigma-Aldrich) in 154 mM NaCl for 15 min at 37°C, as described previously 6. Injury was quantified using a computerized image analysis system (AIS software, Imaging Research, Canada). Alternatively, 4 weeks after ischemia, ischemic volume was evaluated by structural Magnetic Resonance Imaging (MRI) analysis (see below).

**MRI measurements**

**Animal preparation.** The animals were initially anesthetized with isoflurane (5% induction, 1% maintenance) in a 2:1 mixture of N₂/O₂, delivered to the nose cone for spontaneous respiration throughout the experiment. Stereotaxic hear bars were used to minimize movements during the imaging procedure. Temperature was maintained at ~37°C by a feedback-controlled, water circulated heating cradle.

**Data acquisition.** Imaging was performed on a 7T small bore animal Scanner (Bruker Biospec). Two actively coupled radio frequency coils were used: a volume coil of 7.2 cm diameter as transmitter and an anatomically shaped receive-only surface coil array (2x2 array) as receiver. The ParaVision 5.1 software interface (Bruker BioSpin) was also used for data acquisition. The morphological images were obtained with a RARE T2-weighted (T2w) sequence allowing the acquisition of 42 coronal slices of 700 µm thickness, which covered the whole rat brain volume. The morphological images were obtained with an in-plane resolution of
117 x 150 µm (matrix 256 x 200 and a Field of View= 3 x 3 cm); repetition time (TR)=5000 ms, echo time (TE)=36 ms and a RARE factor of 8, for 8 averages.

**Volumetric measurements.** The volume measurements of structural MRI images were obtained manually using custom made software as previously described⁸. For each animal, the regions of interest (ROIs) were manually chosen and drawn on the images for volumetric assessments (Fig. 6d). We computed the lesion volume including the whole infarcted tissue (T2w hyperintense tissue indicative of edema⁹ across different brain areas. This measurement did not include the ventricle volume. Measurements of brain atrophy in cortex, hippocampus and striatum (G. Paxinos and C. Watson, The rat brain in stereotaxic coordinates, 5th ed., Academic Press, New York, 2005) included residual T2w hypointense tissue. Data from each animal were obtained by the integration of averaged ROI area for slice thickness.

**Immunofluorescence and confocal analysis**

Twenty-micron thick coronal sections were used for MBL-A and -C detection in ischemic tissues. Briefly, the sections were rinsed for 1 h in 10% normal goat serum and 0.3% Triton X-100 in 0.1 mol/l PBS at room temperature. The sections were then incubated overnight at 4°C with the primary monoclonal antibodies anti-mouse MBL-A or MBL-C (1:100, Hycult Biotechnology). MBL-A and -C positivity was revealed by Alexa546 fluoro-conjugated goat anti-rat IgG (1:500, Molecular Probes). Brain vessels and nuclei were stained with Alexa488 fluoro-
conjugated isolectin (IB4, 1:200, Invitrogen) and 4'-6-diamidino-2-phenylindole (DAPI, 1µg/ml, Invitrogen) respectively. Appropriate negative controls without the primary antibodies were performed. None of the immunofluorescence reactions revealed unspecific fluorescent signal in the negative controls. Immunofluorescence was acquired using a scanning sequential mode to avoid bleed-through effects by an IX81 microscope equipped with a confocal scan unit FV500 with 3 laser lines: Ar-Kr (488nm), He-Ne red (646nm), and He-Ne green (532nm) (Olympus) and a UV diode. Three-dimensional images were acquired over a 10-12 µm z-axis with a 0.23 µm step size and processed using Imaris software (Bitplane) and Photoshop CS2 (Adobe Systems Europe Ltd) as shown previously.

**Semiquantitative evaluation of the MBL staining.** MBL-A and -C scores were assigned blindly to experimental condition in order to identify different degrees of positivity: - = no positivity, + = low positivity, ++ = intermediate positivity, +++ = high positivity.

**Western blot**

Blood samples were collected at different time points and centrifuged at 2000 g for 15 min at 4°C. Protein concentration of each sample was determined by Bradford method (Protein Assay; Bio-Rad). Equal amounts of protein (10 µg/sample) were electrophoresed on a 5% sodium dodecyl sulphate–polyacrylamide gel and transferred to polyvinyl difluoride membranes. Incubation with primary antibody was performed using anti-C3 polyclonal antibody (1:100;
Santa Cruz Biotechnology) overnight at 4°C followed by 1h incubation with rabbit peroxidase-conjugated secondary antibody (1:2500; Santa Cruz Biotechnology). Immunocomplexes were visualized by chemiluminescence using the ECL Western blot substrate (Pierce, Thermo Scientific). Results were calculated by standardizing the C3 fragments optical density (OD) with the total loaded protein OD (Ponceau solution; Bio-Rad). Quantification of the C3 fragments was carried out using Quantity One Software (Bio-Rad).

**Surface plasmon resonance (SPR)**

Binding studies were carried out using the ProteOn XPR36 Protein Interaction Array system (Bio-Rad Laboratories, Hercules, CA), based on surface plasmon resonance (SPR) technology as shown previously $^{10}$. Recombinant human MBL (rhMBL, R&D Systems, Minneapolis, MN) and bovine serum albumin (BSA) (Sigma) were covalently immobilized onto two parallel flow cell surfaces of the same sensor chip (Bio-Rad), using amine-coupling chemistry, with immobilization levels of about 4000 and 5000 resonance units (RU, 1RU = $\sim$ 1pg protein/mm$^2$), respectively. Polymannosylated dendrimers were injected simultaneously over the two proteins immobilized on the chip and flowed for 3 min at a rate of 30 or 100 µl/min at 25°C. The running buffer was 10mM Tris buffer containing 1.2mM CaCl$_2$, 150 NaCl, and 0.005% Tween 20, pH 7.4. The sensorgrams (time course of the SPR signal in RU) were normalized to a baseline value of 0. The signal observed on the surfaces immobilizing MBL was corrected by subtracting the nonspecific response observed on the reference surface immobilizing BSA. Parallel injections of vehicle alone allowed correction for binding-independent responses.
(i.e., drift effects). The resulting sensorgrams were fitted by the simplest 1:1 interaction model (Langmuir model, analysis software by Proteon, Columbus, OH) to obtain the corresponding association and dissociation rate constants (Kon and Koff).

**Complement hemolytic assay**

Mice were divided in the following groups (n=3/group): 1) WT + saline (vehicle control) and 2) WT + Polyman2. Under isoflurane anesthesia, 50 µl of normal saline (control) or 50 µl saline containing 142 µg Polyman2 was injected intravenously. After 1h, blood was collected via heparinized syringes, centrifuged at 1000 x g for 10 min, aliquoted and frozen at -80°C until performance of the CH50 assay.

The CH50 assay was performed as we have previously described but with modifications. Chicken red blood cells (Colorado Serum in Alsevers buffer) were washed in cold gelatin veronal buffer and sensitized with rabbit anti-chicken RBC (1:1000; Inter-Cell Technologies). The cells were diluted to 1X10⁸/ml. Human serum depleted of complement component 5 (Complement Technologies) was diluted to 20% aliquots to which 1, 3 or 10 µl of plasma from vehicle or Polyman2 treated mice was added as a source of C5. Samples (100 µl each in triplicate) were diluted 1:2 on a 96 well round bottom microtiter plate to a final dilution of 1:1280. Sensitized chicken RBCs (30 µl/well) were added, incubated at 37°C for 30 min on a rocker. RBCs were removed from suspension by centrifugation and the supernatant (85 µl) removed and read at 415 nm. Hemolytic activity was assessed as described.
Statistics

Unpaired t-test was used for functional MBL/MASP-2 complexes, C3 activation fragments, susceptibility of MBL−/− mice (Fig. 2 a,b,c,d), effect of 14C isotype control antibody administration (Fig. 5e). Data in Fig 2b, showing a considerable overlap between groups, were also analyzed by non-parametric Mann Whitney test, confirming a significant difference (p<0.05). Thus, only the result of parametric analysis is reported throughout the text. Unpaired t-test with Welch’s correction was used for ischemic volume 28d after injury in rats (Fig. 6e). One-way ANOVA followed by Dunnett post-hoc test was used for neurological deficits, ischemic volume and C3 activation fragments in Polyman2 experiments (Fig. 4 b,c,f) and for ischemic volume assessment in Ab P2D5 experiment (Fig. 5d). Kruskal-Wallis test followed by Dunn’s test was used for neurological deficits after 3-vo (Fig. 5 a,b,c). Two-way ANOVA for repeated measures followed by Bonferroni post-hoc test was used for neurological deficits (Fig. 6 a,b,c). Two-way ANOVA for matched values (ipsi and contralateral side) followed by Bonferroni post-hoc test was used for MRI cortical and hippocampal structural (T2w) data (Fig. 6 f,g).
Supplemental Figure 1

Representative staining of MBL-A and MBL-C in the ipsilateral cortices 30 min after sham-tMCAo (a) and sham-pMCAo (c). Representative staining of ischemic tissues incubated without primary antibody for MBL-C in tMCAo (b) and pMCAo (d). Nuclei, vessels and MBL (-A or -C) are shown in blue, green and red, respectively. Confocal analysis, scale bar 20µm.
Supplemental Figure 2

Infarct volume assessed 48h after tMCAo in WT and MBL⁻/⁻ (a) indicate that MBL⁻/⁻ mice have a lower susceptibility to tMCAo. Data are expressed as mean±SD, n=9-12 mice, unpaired t-test; **P<0.01. Blood flow measurement in the middle cerebral artery territory in WT and MBL⁻/⁻ mice measured by laser Doppler flowmetry (b). Perfusion rates, recorded before surgery, after common carotid artery and middle cerebral artery occlusion (CCAo and MCAo respectively) and 15 minutes after reperfusion, demonstrate that there is no difference in the blood flow between WT and MBL⁻/⁻ mice at every time point considered. Data are reported as mean±SD, n=3. Two-way ANOVA followed by Bonferroni as post-hoc test.
Supplemental Figure 3

Binding of mannosylated dendrimers to MBL evaluated by SPR, effect of valency. MBL was immobilized on the surface of sensor chip, whereas the dendrimers were injected for three minutes at a flow rate of 100 µl/min. The figure shows the maximal binding (in Resonance Units, RU) obtained when injecting 100 µM of different dendrimers.
**Supplemental Figure 4**

Effect of two different doses of Polyman2 administered at the end of ischemic period on infarct volume 48h after tMCAo. Data are reported as mean±SD, n=10-12. One-way ANOVA followed by Dunnett as post-hoc test; **P<0.01.
**Supplemental Figure 5**

Effect of Polyman2 on brain edema after tMCAo injury. Edema was assessed 48h from tMCAo in mice receiving a single iv administration of vehicle or Polyman2, as described in Fig. 4. Data are expressed as mean+SD. One-way ANOVA followed by Dunnett post-hoc test; *P<0.05.
Supplemental Figure 6

Infarct volume (a) and C3 complement activation fragments (b) assessed 48h after pMCAo in mice receiving a single iv administration of vehicle or Polyman2, 3 or 6h after injury, n=7. Data are expressed as mean±SD. Representative images of MBL-A staining in the ipsilateral cortex 24h after pMCAo in vehicle (left panel) or Polyman2 (post-ischemia treatment time: 6h, right) treated mouse. Nuclei, vessels and MBL stainings in blue, green and red, respectively, n=3 (c). Confocal analysis, scale bar 20µm.
Supplemental Figure 7

Infarct volume assessed 48h after ischemia in the various vehicle groups. **a:** ischemic volumes of mice receiving vehicle 3, 6, 12, 18, 24 or 30h after tMCAo, which were grouped together in the graph shown in Fig. 4c. **b:** ischemic volumes of rats receiving vehicle 20 min before and 6,18 and 24h after 3-vo, which were grouped together in graph shown in Fig. 5d. Dotted lines represent the mean value of all vehicle groups. Data are expressed as mean+SD for each time point.
Supplemental references


