Brain Perivascular Macrophages Initiate the Neurovascular Dysfunction of Alzheimer Aβ Peptides

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ABSTRACT

Rationale: Increasing evidence indicates that alterations of the cerebral microcirculation may play a role in Alzheimer’s disease (AD), the leading cause of late-life dementia. The amyloid-β peptide (Aβ), a key pathogenic factor in AD, induces profound alterations in neurovascular regulation through the innate immunity receptor CD36, which, in turn, activates a Nox2-containing NADPH oxidase leading to cerebrovascular oxidative stress. Brain perivascular macrophages (PVM) located in the perivascular space, a major site of brain Aβ collection and clearance, are juxtaposed to the wall of intracerebral resistance vessels and are a powerful source of reactive oxygen species (ROS).

Objective: We tested the hypothesis that PVM are the main source of ROS responsible for the cerebrovascular actions of Aβ, and that CD36 and Nox2 in PVM are the molecular substrates of the effect.

Methods and Results: Selective depletion of PVM using intracerebroventricular injection of clodronate abrogates the ROS production and cerebrovascular dysfunction induced by Aβ applied directly to the cerebral cortex, administered intravascularly or overproduced in the brain of transgenic mice expressing mutated forms of the amyloid precursor protein (Tg2576 mice). In addition, using bone marrow chimeras we demonstrate that PVM are the cells expressing CD36 and Nox2 responsible for the dysfunction. Thus, deletion of CD36 or Nox2 from PVM abrogates the deleterious vascular effects of Aβ, whereas wild-type PVM reconstitute the vascular dysfunction in CD36-null mice.

Conclusions: The data identify PVM as a previously unrecognized effector of the damaging neurovascular actions of Aβ and unveil a new mechanism by which brain-resident innate immune cells and their receptors may contribute to the pathobiology of AD.

Keywords: Vascular biology, cerebrovascular disease, oxidative stress, endothelium, perivascular macrophages.
Nonstandard Abbreviations and Acronyms:

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>Aβ</td>
<td>Amyloid-β</td>
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<td>ACh</td>
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<td>AD</td>
<td>Alzheimer’s disease</td>
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<td>APP</td>
<td>Amyloid precursor protein</td>
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<td>BM</td>
<td>Bone marrow</td>
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<td>CBF</td>
<td>Cerebral blood flow</td>
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<td>CD33</td>
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<td>Complement receptor type 1</td>
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<td>CBF</td>
<td>Cerebral blood flow</td>
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<td>DiO</td>
<td>DiOC18(3) (3,3'-Dioctadecyloxycarbocyanine Perchlorate</td>
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<td>FITC</td>
<td>Fluorescein</td>
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<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>Glut1</td>
<td>Glucose transporter 1</td>
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<td>Iba1</td>
<td>Ionized calcium binding adaptor molecule 1</td>
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<td>LDU</td>
<td>Arbitrary laser-Doppler perfusion units</td>
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<td>MnTBAP</td>
<td>Mn(III)tetrakis (4-benzoic acid) porphyrin</td>
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<td>Nox2</td>
<td>NADPH oxidase 2</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PVM</td>
<td>Perivascular macrophages</td>
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<td>RFU</td>
<td>Relative fluorescence units</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SNAP</td>
<td>S-Nitroso-N-Acetyl-D,L-Penicillamine</td>
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<td>Tg2576</td>
<td>Mice overexpressing the Swedish mutation of the amyloid precursor protein (APPK670/671L)</td>
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<td>TREM2</td>
<td>Triggering receptor expressed on myeloid cells 2</td>
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<td>WT</td>
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INTRODUCTION

Alzheimer’s disease (AD) is the leading cause of cognitive impairment in the elderly, characterized pathologically by extracellular deposition of the amyloid-β peptide (Aβ) in amyloid plaques and intracellular aggregates of the microtubule associated protein tau (neurofibrillary tangles). Due to demographic shifts and lack of effective treatments, the prevalence of AD is estimated to rise to epidemic proportions in the next few decades and disease-modifying therapeutic interventions are sorely needed.

Several lines of evidence suggest that vascular factors play a pathogenic role in AD. While vascular risk factors increase the risk of AD, cerebrovascular function is altered in pre-symptomatic individuals who will develop AD, suggesting an early involvement in the disease process. The brain is highly dependent on a continuous and well-regulated delivery of cerebral blood flow (CBF), and alterations in cerebral perfusion lead to brain dysfunction and cognitive impairment. A large body of work indicates that Aβ disrupts key mechanisms regulating the cerebral microcirculation. For example, Aβ suppresses the increase in CBF evoked by synaptic activity, a vital homeostatic response that matches oxygen and...
glucose delivery to the energy needs of the active brain, and disrupts endothelial function. These neurovascular alterations are mediated by the innate immunity receptor CD36, polymorphisms of which are linked to AD susceptibility. CD36 binds Aβ and leads to Nox2-dependent production of reactive oxygen species (ROS). However, CD36 and Nox2 are expressed in many cells, including microglia, circulating myeloid cells and endothelium, and the relevant cell type(s) remain to be identified.

The perivascular space (Virchow-Robin space), limited by the glial basement membrane (glia limitans) and the vascular basement membrane, has emerged as a major conduit for brain Aβ clearance, as well as a preferential site of Aβ accumulation. Brain Aβ, released extracellularly during synaptic activity, reaches the perivascular space and exits the brain through different routes. Brain perivascular macrophages (PVM), a unique population of brain myeloid cells distinct from macrophages in heart and systemic vessels, are closely apposed to the wall of cerebral resistance arteries in the perivascular space, and, as all macrophages, can produce large amounts of ROS. Therefore, PVM are exposed to Aβ and are uniquely poised to induce cerebrovascular oxidative stress, but their participation in the vascular effects of Aβ has not been explored.

We examined the role of PVM in the cerebrovascular actions of Aβ. We found that depletion of PVM abrogates the vascular oxidative stress and neurovascular dysfunction induced by Aβ, either administered to wild type (WT) mice or produced endogenously in the brain of transgenic mice expressing a mutated form of amyloid precursor protein (APP) (Tg2576). Furthermore, using bone marrow (BM) chimeras we demonstrated that expression of CD36 and Nox2 in PVM is absolutely required for vascular effects of Aβ. The findings implicate for the first time PVM in the neurovascular dysfunction of Aβ, and suggest a novel mechanism by which brain-resident innate immune cells may promote AD.

METHODS

Mice.

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medicine. Experiments were performed in 3-4 month-old transgenic mice overexpressing the Swedish mutation of the amyloid precursor protein (APP) (Tg2576) or age-matched WT littermates, referred to as WT mice. In BM chimera experiments CD36-/- and Nox2-/- mice were used as BM donors. In some studies, GFP+ mice (JAX Stock #006567) were used as BM donors. All mice were males and derived from in house colonies.

Intracerebroventricular injection of clodronate or dextran

Liposomes containing clodronate or phosphate buffered saline (PBS) were administered intracerebroventricularly (icv) as previously described. Isoflurane-anesthetized mice were placed in a stereotaxic frame. Ten µl of clodronate-liposomes (7 mg/ml) or PBS-liposomes (vehicle) were injected into the cerebral ventricles with a glass micropipette through a burr hole drilled on the right parietal bone. Mice were used in the experiments 5-7 days later, when PVM depletion is well developed and stable. For dextran injections, 10 µl of Alexa Fluor® 680 dextran (10,000 MW, anionic, fixable, ThermoFisher Scientific, D34680; 2.5 mg/ml) in saline or saline alone were injected and PVM labeling was examined 24 hrs later.

Labeling cortical blood vessels with DiO.

Cortical blood vessels were labeled with the lipophilic dye DiO [DiOC18(3) (3,3'-Dioctadecyloxacarbocyanine Perchlorate)], as described. Briefly, mice were anesthetized (5% isoflurane) and transcardially perfused with PBS (2 ml) followed by DiO (1:50, V-22886, Molecular Probes;
5ml/mouse) and then by 4% paraformaldehyde (PFA). Brains were harvested and post-fixed in 4% PFA overnight, and then cut (thickness 150 µm) using a vibratome and examined under the confocal microscope (Leica SP5).

**Immunohistochemistry.**
Mice were anesthetized with sodium pentobarbital (120 mg/kg, i.p.) and perfused transcardially with PBS followed by 4% PFA in PBS. Brains were removed, stored overnight, and sectioned in a vibratome (section thickness: 40 µm). Free-floating brain sections were permeabilized with 0.5% Triton X-100 and non-specific binding was blocked with 1% of normal donkey serum. Sections were randomly selected and incubated with the primary antibodies CD206 (clone MR5D3, rat polyclonal, 1:200, Serotec), Glut-1 (rabbit polyclonal, 1:200, Calbiochem), Iba-1 (rabbit polyclonal, 1:500, Wako Chemicals), or GFAP (mouse monoclonal, 1:1000, Sigma) overnight at 4°C. After washing, brain sections were incubated with a Cy5- or a FITC-conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories), mounted on slides and imaged with a confocal microscope (Leica SP5). The specificity of the immunofluorescence was verified by omission of the primary and/or secondary antibody or blocking of the antigen. All quantifications were performed by investigators blinded to the treatment on randomly selected fields within the somatosensory cortex.

**Identification and quantification of PVM in somatosensory cortex.**
PVM were identified by well-established criteria, including expression of CD206, ability to phagocytize dextran and perivascular location34, 38, 41 (fig 1). The association with cortical blood vessels was confirmed by co-labeling with the endothelial marker Glut-1 (rabbit polyclonal, 1:200, Calbiochem), the smooth muscle marker α-actin or DiO40. For CD206+ PVM, randomly selected fields (20x objective; 4 confocal images/mouse; n=5 mice/group) within the somatosensory cortex were analyzed. For dextran+ PVM, a representative coronal section from each mouse was reconstructed from tiled images taken with the confocal microscope, and the whole somatosensory cortex (n=3/group) was analyzed. ImageJ (NIH) was used for all image analyses.

**Cerebrovascular ROS measurement.**
ROS production was assessed in vivo by hydroethidine (HE) microfluorography23-25, 34, 42. To assess ROS production in PVM, mice were first injected icv with clodronate- or PBS-liposome and 5-7 days later with dextran (see above). The day after dextran injection, in WT mice HE (2 µM in Ringer; Invitrogen) with or without Aβ (5 µM) was superfused over the somatosensory cortex. In tg2576 mice, HE (10 mg/kg) was injected into the jugular vein. BM-transplanted mice were injected with icv dextrans one day before ROS measurement. Sixty minutes after HE administration, mice were injected with DiO to label cerebral blood vessels as described above. Coronal brain sections were then cut through the cortex underlying the cranial window, and ROS dependent fluorescence associated with blood vessels or PVM was quantified as described previously23-25, 34, 42.

**Bone marrow transplant.**
Procedures for BM transplant have been previously described34, 43 and are only summarized. Whole-body irradiation was performed in 7 weeks-old mice (Nordion Gammacell 40 Exactor). Eighteen hours later, mice were transplanted with BM cells (2x10⁶, i.v.) isolated from the donor CD36-/-, Nox2-/-, and WT controls. Mice were housed in cages with sulfamethoxazole (0.12%; w/v) and trimethoprim (0.024%) added to drinking water for the first 2 weeks. Reconstitution of BM cells was verified 5 weeks after irradiation by testing the percentage of positive CD36 or Nox2 genomic DNA in isolated blood leukocytes43. Chimerism was >95% for both CD36-/- and Nox2-/- BM chimeras. For studies of PVM number and distribution after BM transplant in Tg2576 mice, the BM of transgenic mice expressing GFP was transplanted into irradiated Tg2576 mice or littermates.
General surgical procedures for CBF measurement.
As described in detail elsewhere, mice were anesthetized with isoflurane (induction, 5%; surgery, 2%) and maintained with urethane (750 mg/kg; i.p.) and α-chloralose (50 mg/kg; i.p.). A femoral artery was cannulated for recording of arterial pressure and collection of blood samples for blood gas analysis. The trachea was intubated and mice were artificially ventilated with a mixture of N₂ and O₂. Arterial blood pressure (80-90 mmHg), blood gases (pO₂, 120-140 mmHg; pCO₂, 30-40 mmHg; pH, 7.3-7.4), and rectal temperature (37°C) were monitored and controlled. Throughout the experiment the level of anesthesia was monitored by testing motor responses to tail pinch. The somatosensory cortex was exposed through a small craniotomy (2x2 mm). The dura was removed, and the exposed cortex was continuously bathed with a modified Ringer’s solution (36-37°C; pH: 7.3-7.4) (see ref. for composition). CBF was continuously monitored at the site of superfusion with a laser-Doppler probe (Vasamedic, St. Paul, MN) positioned stereotaxically on the neocortical surface and connected to a computerized data acquisition system. CBF values were expressed as percent increase relative to the resting level. Resting CBF is reported as arbitrary laser-Doppler perfusion units (LDU). Zero values for CBF were obtained after the heart was stopped by an overdose of isoflurane at the end of the experiment.

Experimental protocol for CBF experiments.
CBF recordings were started after arterial pressure and blood gases were in steady state; CBF responses to whisker stimulation were recorded while gently stroking the whiskers with a cotton-tipped applicator for 60 sec. All pharmacological agents were dissolved in a modified Ringer’s solution. To test endothelium-dependent responses, the endothelial nitric oxide (eNOS)-dependent vasodilator acetylcholine (10 µM, Sigma), the Ca²⁺ ionophore A23187 (3 µM; Sigma) or bradykinin (50 µM; Sigma) was topically superfused for 3-5 min and the evoked CBF increases recorded. To test smooth muscle reactivity, CBF response to adenosine (400 µM, Sigma) or the NO donor S-Nitroso-N-acetyl-DL-penicillamine (SNAP; 50 µM, Sigma) were examined. The increase in CBF produced by hypcapnia was tested by introducing 5% CO₂ in the ventilator to increase arterial pCO₂ up to 50-60 mmHg. Once a stable increase in CBF was obtained for 3-5 min, pCO₂ was returned to normocapnia.

Measurement of Aβ.
Aβ was measured using an ELISA-based assay, as described previously. Briefly, the left hemispheres from the mice used for CBF studies were sonicated in 1% SDS with protease inhibitors and centrifuged. The supernatant contained SDS-soluble Aβ peptides. The pellet was sonicated in 70% formic acid and centrifuged as above. The formic acid extract was neutralized by a 1:20 dilution into 1 M Tris phosphate buffer (pH 8.0). Aβ1-40 and Aβ1-42 concentrations (pM) were determined in supernatant (SDS-soluble) and in the formic acid extract of the pellet (SDS-insoluble) using the BAN-50/BA-27 and BAN-50/BC005 sandwich ELISA assay.

Statistics.
Sample size was determined by power analysis based on previous published works published by our lab on CBF regulation. Animals were randomly assigned to treatment or control group and analysis was performed in a blinded fashion. Group difference was analyzed using Student’s t test (paired or unpaired) or analysis of variance (ANOVA) with Tukey’s test, as appropriate. Data are expressed as mean±SEM and statistical difference was considered significant at p<0.05.
RESULTS

Brain PVM depletion ameliorates the neurovascular dysfunction induced by Aβ.

PVM, identified by CD206 immunochemistry, their perivascular location and their ability to phagocytize dextran, were observed in close apposition to somatosensory cortex blood vessels (Figure 1; Online Figure I). In particular, PVM were juxtaposed to penetrating arterioles identified by the smooth muscle cell marker α-actin (Online Figure I), attesting to their association with cerebral resistance vessels. PVM also expressed CD36 (Online Figure II), the innate immunity receptor involved in the cerebrovascular effects of Aβ25, 26. Consistent with previous observations34, PVM were not observed in vessels smaller than 10 µm (precapillary arterioles and capillaries), due to the fusion of the glial and vascular basement membranes and obliteration of the perivascular space29.

To deplete brain PVM we used icv injection of liposome-encapsulated clodronate and assessed the extent of the depletion 5 days later, when the effect is most pronounced and stable34, 38. Clodronate, but not control liposomes, deplete PVM and meningeal macrophages in the somatosensory cortex (>80%). In agreement with previous observations in adult mice34, clodronate did not deplete microglia, smooth muscle cells, astrocytes, or endothelium (Figure 1; Online Figures. I-III). In WT mice treated with control liposomes, superfusion of Aβ1-40 (5µM) on the somatosensory cortex reduced resting CBF and attenuated the CBF increase induced by whisker stimulation (functional hyperemia; -40±3%), by neocortical application of the endothelium-dependent vasodilators ACh (-31±3%), bradykinin (-46±1%), and Ca2+ ionophore A23187 (-34±6%), or by the NO donor SNAP (-29±3%) (Figure 2; Online Figure IVA-C) (p<0.05). As before23-26, the CBF response to neocortical application of the smooth muscle relaxant adenosine or to hypercapnia, a potent cerebrovasodilatatory stimulus45, 46, was not affected (Figure 2D; Online Figure IVD) (p>0.05). However, we cannot rule out that a reduction would be observed with a milder hypercapnic challenge producing smaller CBF increases. PVM depletion by clodronate, did not alter CBF responses, but prevented in full the Aβ-induced attenuation in functional hyperemia, endothelium-dependent responses, and response to SNAP (Figure 2A-C; Online Figure IVA-C). Clodronate also attenuated the increase in cerebrovascular ROS induced by Aβ (Figure 2E).

To provide further evidence that PVM participate in the neurovascular effects of Aβ, we used 3-4 month-old Tg2576 mice23, 25, 42. At this age, Tg2576 mice have elevated levels of brain Aβ but do not exhibit amyloid pathology (amyloid plaques and cerebral amyloid angiopathy)23-26, 42. The number of PVM did not differ between Tg2576 mice and WT littermates (Figure 1E), but the intensity of the CD36 immunolabel seemed greater in CD206+ PVM of Tg2576 mice (Online Figure IIIB), as reported25, 26. Clodronate depleted PVM in Tg2576 mice as effectively as in WT mice (Figure 1; Online Figure IIIB), without altering brain Aβ1-40 and Aβ1-42 (Figure 3D). However, in agreement with the results with topical Aβ, clodronate treatment prevented the neurovascular dysfunction and vascular oxidative stress in Tg2576 mice (Figure 3A,B; Online Figure IVE-I). These observations indicate that PVM depletion ameliorates the cerebrovascular dysfunction induced by exogenous Aβ and observed in Tg2576 mice.

Deletion of CD36 or Nox2 in PVM ameliorates the neurovascular dysfunction.

We used CD36−/− or Nox2−/− BM chimeras to replace PVM with PVM lacking CD36 or Nox2 in WT and Tg2576 mice. After lethal irradiation, PVM are eliminated and slowly replaced with BM-derived macrophages, but microglia and other resident brain cells are not affected, at least in young WT and APP mice34, 47. This approach has been previously used for gene targeting in PVM34, 47. Since immune cell trafficking may be altered in APP-expressing mice36, 47, 48, we first investigated whether the repopulation of the perivascular space by PVM after BM transplant was comparable in WT and Tg2576 mice. GFP+ BM was transplanted in WT and Tg2576 mice and, 2 months later, perivascular GFP+ cells were examined in
both groups. We found that the number of PVM and their perivascular distribution did not differ between WT and Tg2576 mice (Online Figure V). Furthermore, to investigate if PVM repopulation is altered by lack of CD36 or Nox2 in macrophages, we quantified PVM in tg2676 mice transplanted with CD36-/- or Nox2-/- BM, and there were no differences in number and distribution of PVM (Figure 4A-E).

Next, we studied the cerebrovascular dysfunction induced by Aβ in BM chimeras. Functional hyperemia, endothelium-dependent CBF responses, and responses to adenosine, SNAP or hypercapnia in WT mice receiving WT BM (WT➔WT) were identical to those observed in naïve WT mice (Figure 5A-D; Online Figure VIA-D), demonstrating that the BM transplantation procedure did not alter baseline cerebrovascular responses. However, the neurovascular dysfunction and vascular oxidative stress induced by topical application of Aβ1-40 were not observed in CD36-/-➔WT or Nox2-/-➔WT chimeras (Figure 5A-E; Online Figure VIA-D). Importantly, transplant of WT (CD36+/+) BM in CD36-/- mice was able to re-establish the neurovascular dysfunction induced by Aβ1-40 (Figure 5F-G). The effect was counteracted by neocortical superfusion of the ROS scavenger MnTBAP (Figure 5F-G; Online Figure VIE-F), consistent with the involvement of ROS in the dysfunction induced by the BM-derived PVM repopulating the cerebral vasculature after transplant.

In agreement with the Aβ superfusion findings, transplantation with CD36-/- or Nox2-/- BM rescued the neurovascular dysfunction in Tg2576 mice, without altering brain Aβ levels (Figure 6; Online Figure VII). Transplant of WT BM in Tg2576 mice did not suppress ROS production in PVM, but transplantation of CD36-/- or Nox2-/- BM abolished it (Figure 4), attesting to the role of CD36 and Nox2 in PVM in the Aβ-induced ROS production.

PVM depletion ameliorates the neurovascular dysfunction induced by circulating Aβ.

Circulating and brain Aβ both have a role in neurovascular dysfunction, an effect mediated by ROS. Circulating Aβ is thought to cross the BBB by engaging the receptor for advanced glycation end-products (RAGE). Therefore, we sought to determine if PVM also contribute to the neurovascular effects of circulating Aβ. To this end, first we sought to establish if blood borne Aβ enters the perivascular space and reaches PVM. Intracarotid administration of cy5-labelled Aβ1-40, at a concentration and infusion rate that produce a steady state increase in plasma Aβ comparable to that of Tg2576 mice, was able to cross the vascular wall, enter the perivascular space, and reach PVM (Figure 7A,B; Online Figure VIII). Clodronate depleted PVM without affecting Cy5-labelled Aβ1-40 (Figure 7C,D). As previously reported, Aβ1-40 infusion resulted in attenuation of the CBF increase produced by whisker stimulation and by endothelium-dependent vasodilators in the somatosensory cortex ipsilateral to the infusion, while endothelium-independent responses were not affected (Figure 7F). PVM depletion prevented in full the neurovascular dysfunction induced by circulating Aβ1-40, an effect associated with suppression of vascular oxidative stress (Figure 7E,F).
DISCUSSION

Several studies have shown that Aβ leads to vascular dysfunction through oxidative stress\textsuperscript{16, 17, 20}, but the effector cells exposed to Aβ and responsible for the ROS production were not identified. Since brain PVM can produce large amounts of ROS\textsuperscript{34} and are located in the perivascular space, a major site of brain Aβ trafficking and accumulation\textsuperscript{31, 33}, we sought to determine whether PVM are involved in the neurovascular effects of the peptide. We found that selective depletion of PVM abrogates the vascular oxidative stress and neurovascular dysfunction induced by Aβ, either applied directly to the neocortex of WT mice or produced endogenously in Tg2576 mice. The effect could not be attributed to depletion of microglia, to non-specific alterations in vascular structure and reactivity resulting in vasoparalysis, or to reductions in brain Aβ in Tg2576 mice. Elevated levels of circulating Aβ have been implicated in vascular alterations in AD and other dementias\textsuperscript{51-54}, and lead to cerebrovascular disturbances in animal models\textsuperscript{40}. Therefore, we asked if PVM also contribute to the deleterious neurovascular effects of circulating Aβ. We found that intravascular Aβ crosses the vascular wall, enters the perivascular space, reaches PVM, induced ROS production, and alters neurovascular function. These novel observations establish PVM as the main source of the vascular ROS responsible for the CBF alterations induced by parenchymal and plasma Aβ.

Next, we sought to establish whether CD36 and Nox2 in PVM are required for the deleterious cerebrovascular effects of Aβ. To this end, we used BM chimeras in which CD36 or Nox2 was deleted in PVM by transplantation of CD36- or Nox2-deficient BM. First, we verified that the extent of PVM replacement by blood-borne myeloid cells was comparable between the two mouse lines and was not altered by lack of CD36 or Nox2. Then, we tested the cerebrovascular effects of Aβ in the chimeras. The BM transplant procedure did not alter CBF regulation in WT mice or the suppression of neurovascular responses in Tg2576 mice. However, transplantation of CD36- or Nox2-deficient BM abrogated the cerebrovascular dysfunction and vascular oxidative stress in WT mice exposed to exogenous Aβ or in Tg2576 mice. Furthermore, WT BM was able to re-establish Aβ-induced vascular dysfunction in CD36-deficient mice, suggesting that the CD36 signaling pathway in PVM is absolutely necessary for the harmful vascular effects of Aβ. The findings, collectively, identify for the first time CD36 and Nox2 localized to PVM as the molecular substrates underlying the neurovascular dysfunction induced by Aβ.

Although cerebral endothelial cells in culture have the potential to produce ROS in response to Aβ via CD36\textsuperscript{42}, depletion of PVM completely abrogates vascular oxidative stress and neurovascular dysfunction, suggesting an essential role of PVM in the neurovascular effects of Aβ in vivo. Therefore, previous results in which Aβ vasoactivity was not observed in CD36-null mice\textsuperscript{25} have to be attributed to lack of CD36 in PVM and not endothelial cells. This conclusion is also supported by our finding that WT BM transplant in CD36\textsuperscript{-/-} mice, in which endothelial cells lack CD36, re-establishes the ROS-dependent neurovascular dysfunction induced by Aβ. Therefore, WT PVM are able to drive the neurovascular dysfunction even if endothelial cells lack CD36. Aβ has also been reported to increase ROS in isolated cerebral blood vessels\textsuperscript{25, 55}. However, these vascular preparations are likely to also include PVM which remain attached to the outer vessel wall during the isolation procedure.

There has been an increasing appreciation for the role of PVM in the brain’s own innate immunity system\textsuperscript{35, 36}. PVM, a unique population of brain myeloid cells distinct from microglia, have been implicated in hypothalamic-adrenal axis and neurohumoral activation in the setting of myocardial infarction, emotional stress or systemic inflammation\textsuperscript{56-58}. PVM may also have a role in neuroimmune trafficking in brain infections\textsuperscript{38, 58}, and in the structural and functional cerebrovascular alterations of hypertension\textsuperscript{34, 59}, as well as in tumor angiogenesis and cerebrovascular repair\textsuperscript{40}. In models of cerebral amyloidosis, PVM depletion reduces Aβ accumulation in cerebral blood vessels\textsuperscript{61}. The present results expand our understanding of the pathobiology of these cells in AD by revealing a key role of PVM in Aβ-induced neurovascular dysfunction.
The perivascular space and associated innate immune cells have recently attracted much attention in the mechanisms of AD\(^33,62\). The brain’s perivascular space is a major conduit for clearance of toxic molecules from the brain, including A\(\beta\)\(^{33,63}\). In AD, tortuous perivascular spaces may impede A\(\beta\) clearance from the brain and promote the harmful interaction between A\(\beta\) and cerebral blood vessels, as well as cerebral amyloid angiopathy\(^{30,31,64}\). Our results provide the first demonstration that PVM are the cells in the perivascular space driving the neurovascular dysfunction induced by A\(\beta\). On the other hand, increasing evidence suggests a role of innate immunity cells and receptors in AD pathogenesis\(^{62}\). While microglial cells, resident brain immune cells, may degrade A\(\beta\) and shield the brain from the deleterious effects of amyloid\(^{62,65}\), innate immunity receptors, including CR1, TREM2, CD33, and CD36, among others, have been linked to increased AD susceptibility in genome-wide association studies\(^{22,62}\). However, it remained unclear how innate immunity exerts its harmful effects in AD\(^{36}\). Our findings identify PVM as critical cells initiating A\(\beta\)-induced neurovascular dysfunction and suggest a new mechanism by which innate immune cells in the perivascular space may contribute to AD.

A limitation of the present study is that, by using presymptomatic Tg2576 mice, we could not assess the impact of PVM on the cognitive deficits associated with A\(\beta\) accumulation. This is for the following reasons. Considering that the effect of clodronate is limited to a few weeks\(^{38}\), we could not determine if PVM depletion protects Tg2576 mice from cognitive deficits, which develop over several months\(^{24,26,37}\). We also explored the possibility of using BM chimeras to delete Nox2 and CD36 in PVM. However, this approach proved not to be feasible because, as the Tg2576 BM chimeras age, the brain parenchyma is infiltrated by BM-derived cells with the morphological characteristics of microglia (unpublished observations)\(^{47}\). Therefore, the specific contribution of PVM replacement could not be reliably assessed under these conditions. These limitations notwithstanding, considering the well-established link between neurovascular dysfunction and cognitive impairment in this mouse model\(^{24,26}\), it is reasonable to postulate that PVM depletion may result in improved cognition. However, this assumption needs to be verified experimentally. Future studies using more suitable methods to selectively target PVM in older APP mice could assess the role of PVM on cognitive dysfunction, as well in the phosphorylation of tau, a major pathogenic factor in AD which is influenced by endothelial NO\(^{66}\).

In conclusion, we have demonstrated that PVM are essential for the neurovascular dysfunction induced by A\(\beta\) in three separate mouse models. PVM exert their harmful effects through the A\(\beta\) “receptor” CD36 and the ROS producing enzyme Nox2. PVM are also needed for the neurovascular dysfunction induced by circulating A\(\beta\), an effect mediated by A\(\beta\) entering the perivascular space and reaching PVM to induce vascular oxidative stress. The findings identify PVM as the missing link between A\(\beta\) and neurovascular dysfunction, and unveil a new mechanism by which innate immune cells and receptors, long thought to play a role in the disease, contribute to the pathobiology of AD. Therefore, manipulating PVM could be a promising therapeutic strategy to stave off A\(\beta\)-induced brain dysfunction and damage.

**SOURCES OF FUNDING**

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**DISCLOSURES**

None.
REFERENCES


FIGURE LEGENDS

**Figure 1:** PVM, juxtaposed to penetrating cerebral blood vessels, are depleted by clodronate. In WT mice (non-transgenic littermates) injected with PSB-liposome (vehicle), PVM identified by phagocytized dextran (green) are closely apposed to penetrating neocortical vessels, identified by the lipophilic dye DiO (red) (A). PVM in Tg2576 mice injected with vehicle have numbers and distributions similar to WT mice (B). Intracerebroventricular (icv) administration of clodronate-containing liposomes depletes PVM both in WT and Tg2576 mice 5 days later (C,D). Quantification of the number of PVM in WT and tg2576 mice after icv injection of liposomes containing PBS or clodronate (E). No differences between WT and Tg2576 mice are observed. *p<0.05 from PBS; n=5/group; Analysis of variance and Tukey’s test.

**Figure 2:** PVM depletion prevents the cerebrovascular effects of neocortical superfusion of Aβ1-40 in WT mice. In WT mice treated with PBS liposomes, superfusion of the somatosensory cortex with Aβ1-40 (5µM) attenuates resting CBF (A), and the increase in CBF evoked by whisker stimulation (B), or by neocortical application of ACh (10µM) (C), but not adenosine (400µM) (D). Treatment with clodronate does not affect baseline cerebrovascular responses, but rescues the attenuation in resting CBF and CBF responses to whiskers stimulation and ACh induced by Aβ1-40 superfusion. Furthermore, clodronate suppresses the Aβ-induced increase in cerebrovascular ROS in the somatosensory cortex, assessed by DHE microfluorography (E). LDU, arbitrary laser-Doppler perfusion units; RFU, relative fluorescence units; *p<0.05 from vehicle, ANOVA and Tukey’s test; n=5/group.

**Figure 3:** PVM depletion counteracts the neurovascular dysfunction and vascular oxidative stress in Tg2576 mice. PVM depletion by clodronate counteracts the attenuation in the CBF response to whisker stimulation (A) or neocortical application of ACh (B), as well as the increase in cerebrovascular ROS (C) in Tg2576 mice. Clodronate does not alter brain concentrations of SDS-soluble Aβ1-40 and Aβ1-42 in Tg2576 mice. RFU, relative fluorescence units; *p<0.05 from WT, ANOVA and Tukey’s test; n=5-6/group.

**Figure 4:** Effect of transplantation of CD36-/- or Nox2-/- BM in Tg2576 mice on PVM number and ROS production. Vessels are labeled with DiO (red), ROS production is detected with DHE (blue) and PVM are labeled with dextran (green). In WT mice transplanted with WT BM (WT➔WT) PVM have numbers and distribution comparable to those of naïve WT mice (A). Similarly, Tg2576 mice transplanted with WT BM (WT➔Tg) exhibited number and distribution similar to those of naïve Tg2576 mice (B). However, ROS production in PVM was observed in WT➔Tg mice (DHE insert in B). Transplant of CD36-/- (CD36➔Tg ) or Nox2-/- (Nox2➔Tg) BM in Tg2576 mice does not alter PVM number or distribution, but suppresses PVM ROS production (DHE inserts in C and D). Quantification of the numbers of PVM in the BM chimeras studied (E). Quantification of cerebrovascular ROS in the BM chimeras showing that ROS production is suppressed in CD36➔Tg and Nox2➔Tg mice (F). RFU, relative fluorescent units; *p<0.05 from WT➔WT, CD36➔Tg, or Nox2➔Tg; ANOVA and Tukey’s test; n=5/group.

**Figure 5:** Deletion of CD36 or Nox2 in PVM ameliorates the neurovascular dysfunction and vascular oxidative stress induced by Aβ1-40 superfusion in WT mice. In WT➔WT mice, cerebrovascular response to whisker stimulation, ACh, and adenosine do not differ from those of naïve WT mice (A-D). Furthermore, Aβ1-40 superfusion attenuates resting CBF and CBF responses to whisker stimulation and ACh, but not adenosine (A-D). However, these cerebrovascular effects of Aβ1-40 are not observed in CD36➔Tg and Nox2➔Tg chimeras (A-D). Aβ1-40 increases cerebrovascular ROS production WT➔WT, but not in CD36➔Tg and Nox2➔Tg mice (E). Transplant of WT (CD36+/+) BM is able to re-establish the neurovascular dysfunction induced by Aβ in CD36+/- mice (WT➔CD36+/-), an effect counteracted by...
neocortical superfusion of the ROS scavenger MnTBAP (50µM) (F,G). RFU, relative fluorescence units; *p<0.05 from vehicle (A-D), CD36➔WT, or Nox2➔WT (F,G); ANOVA and Tukey’s test; n=5-6/group.

**Figure 6:** Deletion of CD36 or Nox2 in PVM counteracts the neurovascular dysfunction and vascular oxidative stress in Tg2576 mice. Tg2576 mice receiving WT BM (WT➔Tg) exhibit attenuation in CBF responses to whisker stimulation (A,B) and ACh (C) similar to those observed in Tg2576 mice not subjected to BM transplantation. Transplant of CD36/-/- or Nox2/-/- BM rescues the neurovascular dysfunction in Tg2576 mice (A-D), without altering brain Aβ1-40 and Aβ1-42 (E,F). *p<0.05 from WT➔WT, CD36➔Tg, or Nox2➔Tg; ANOVA and Tukey’s test; n=5-6/group.

**Figure 7:** Circulating Aβ1-40 reaches PVM and mediates oxidative stress and neurovascular dysfunction. In WT mice treated with PBS, Cy5-labelled Aβ1-40 infused into the carotid artery (Cy5-Aβ1-40) (1µM, 150 µl/hr) (blue) colocalizes with dextran-labelled PVM (green) surrounding cerebral blood vessels labelled with DiO (red), and resulting in the cyan color in the merged image (A). Orthogonal projections illustrating co-localization of dextran-labelled PVM (green) and Cy5-labelled Aβ (blue) around blood vessels (red) (B). Cy5-labelled Aβ is colocalized with more than 80% of dextran-positive PVM (C). In mice treated with clodronate, the signal from Cy5-labelled Aβ1-40 is still present in the brain and not different from PBS treated mice (D), but is no longer associated with PVM, which are depleted (Online Figure VIII). ROS are increased in PVM of mice receiving the i.c. infusion of Aβ1-40 (icAβ1-40), which is suppressed by clodronate treatment (E). icAβ1-40 attenuates the increase in CBF induced by whisker stimulation or ACh, an effect reversed by clodronate (F). Resting CBF or the CBF response to adenosine is not affected by icAβ (F). LDU, arbitrary laser-Doppler perfusion units; RFU relative fluorescence units; *p<0.05 from vehicle, ANOVA and Tukey’s test; n=5-6/group.
NOVELTY AND SIGNIFICANCE

What Is Known?

- Alzheimer’s disease (AD), the most common cause of late-life dementia, is characterized pathologically by the accumulation in brain of the amyloid-b peptide (Ab) and the microtubule associated protein tau
- Alterations in the regulation of the cerebral microcirculation have recently emerged as a significant contributor to cognitive impairment in AD
- The neurovascular dysfunction is mediated by reactive oxygen species (ROS) produced by the binding of Ab to the innate immunity receptor CD36 leading to activation of the superoxide producing enzyme Nox2

What New Information Does This Article Contribute?

- Perivascular macrophages (PVM), myeloid cells juxtaposed to the outer wall of arteriole penetrating the substance of the brain, are the main source of the ROS mediating the neurovascular dysfunction produced by brain Ab
- PVM are also responsible for neurovascular alterations mediated by plasma Ab, which crosses the blood-brain barrier and reaches these cells in the perivascular space
- PVM are the cellular site of the expression of CD36 and NOX2 responsible for neurovascular dysfunction

Alterations in the regulation of cerebral microvasculature play a pathogenic role in AD. Such neurovascular dysfunction may deprive the brain of oxygen and glucose, amplifying the harmful neuronal effects of Aβ and tau pathology. The Aβ peptide in brain and plasma alters neurovascular function through CD36 and Nox2-derived ROS, but the cellular bases of the effect have not been elucidated. We report that brain PVM, a unique sub-population of resident brain macrophages exposed to Aβ draining through the perivascular space, are necessary and sufficient for the oxidative stress driving the neurovascular dysfunction in mouse models of amyloid pathology. Furthermore, we identify PVM as the cells expressing CD36 and Nox2 responsible for the vascular effects of Aβ. These observations establish PVM as the main source of the ROS responsible for the CBF alterations induced by Aβ, and identify these cells as the site of CD36 and Nox2 expression enabling the neurovascular dysfunction. The results expand our understanding of the role of PVM in brain diseases, suggest a new way in which innate immune cells may contribute to AD, and provide a new putative therapeutic target for this devastating disease.
**FIGURE 2**

**A** Resting CBF

- Vehicle
- Aβ_{1-40}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CBF (LDU)</th>
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<tr>
<td>PBS</td>
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<tr>
<td>Clodronate</td>
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**B** Whisker stimulation

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<td>Clodronate</td>
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**C** Acetylcholine

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<td>Clodronate</td>
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**D** Adenosine

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<td>Clodronate</td>
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**E** ROS

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<tr>
<td>Clodronate</td>
<td>5 ± 2</td>
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* denotes statistical significance.
FIGURE 5

A

Resting CBF

Vehicle  Aβ₁₋₄₀

WT→WT  CD36→WT  Nox2→WT

CBF (% increase)

B

Whisker stimulation

WT→WT  CD36→WT  Nox2→WT

CBF (% increase)

C

Acetylcholine

WT→WT  CD36→WT  Nox2→WT

CBF (% increase)

D

Adenosine

WT→WT  CD36→WT  Nox2→WT

CBF (% increase)

E

ROS

WT→WT  WT→WT  CD36→WT  Nox2→WT

RFU (x10⁶)

F

Whisker stimulation

(WT→CD36⁺/+)

Vehicle  Aβ₁₋₄₀  MnTBAP

WT→WT  WT→WT  CD36→WT  Nox2→WT

CBF (% increase)

G

ACh

(WT→CD36⁻/−)

Vehicle  Aβ₁₋₄₀  MnTBAP

WT→WT  WT→WT  CD36→WT  Nox2→WT

CBF (% increase)
FIGURE 6

A. Whisker stimulation

B. CBF (% increase) over time

C. Acetylcholine

D. Adenosine

E. Brain Aβ1-40

F. Brain Aβ1-42

<table>
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<th>WT→Tg</th>
<th>CD36→Tg</th>
<th>Nox2→Tg</th>
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Graphs show the percentage increase in CBF and brain Aβ concentrations under different conditions.
Brain Perivascular Macrophages Initiate the Neurovascular Dysfunction of Alzheimer Aβ Peptides
Laibaik Park, Ken Uekawa, Lidia Garcia-Bonilla, Kenzo Koizumi, Michelle Murphy, Rose Pitstick, Linda H Younkin, Steven G Younkin, Ping Zhou, Gerge A Carlson, Josef Anrather and Costantino Iadecola

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http://circres.ahajournals.org/content/suppl/2017/05/17/CIRCRESAHA.117.311054.DC1

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http://circres.ahajournals.org//subscriptions/
Online Figure I: PVM are associated with resistance arterioles: CD206+ PVM are juxtaped along penetrating arterioles identified by the smooth muscle cell marker α-actin (A), both in WT and in Tg2576 mice (B). Clodronate depletes PVM but does not affect the smooth muscle cell marker α-actin (B). α-actin immunoreactivity, is not affected by icv injection of clodronate in WT and tg2576 mice (B). RFU, relative fluorescence units p>0.05, student’s t-test; n=5/group.
Online Figure II: CD36 expression in CD206+ PVM and co-localization between CD206 and dextran in WT and Tg2576 mice. CD206-labelled PVM also expressed CD36, both in WT and Tg2576 mice (A,B). Merged images illustrating that clodronate depletes CD36/CD206 double-positive cells both in WT and Tg2576 mice (B). More than 90% of dextran-positive cells also express CD206 (C). * p<0.05, analysis of variance and Tukey's test; n=5/group.
Online Figure III: Clodronate does not affect cerebral microvessels, Iba1+ macroglia/macrophage, and astrocytes. Microvessels identified by the endothelial marker Glut1+(A), microglia/macrophages (Iba1+)(B), and astrocytes (GFAP+)(C) in somatosensory cortex are not affected by clodronate-liposomes, both WT and tg2576 mice. p>0.05, analysis of variance and Tukey’s test; n=5/group.
Online Figure IV: Effect of clodronate on cerebrovascular responses in WT mice with Aβ₁-40 superfusion, and in Tg2576 mice. The attenuation of cerebrovascular response to topical application of bradykinin (A), the calcium ionophore A23187 (B), or the NO donor SNAP (C) induced by neocortical superfusion of Aβ₁-40 is not observed in WT mice treated with clodronate. The CBF response to hypercapnia (pCO₂ 50-60 mmHg) is not affected by Aβ₁-40 or clodronate treatment (D). Clodronate also rescues CBF responses in Tg2576 mice (E-G). The CBF response to hypercapnia or adenosine is not attenuated in Tg2576 mice and is not altered by clodronate (H,I). *p<0.05 from vehicle or WT, ANOVA and Tukey’s test; n=5-6/group.
Online Figure V: Number of perivascular distribution of GFP+ cells after GFP+ BM transplant in WT and Tg2576 mice: WT and Tg2576 mice were transplanted with GFP+ BM and the repopulation of the perivascular space of PVM was determined and quantified 2 months later. The pattern of perivascular localization (A,B) and number (C) of GFP+ PVM is comparable in both groups. p>0.05, student’s test; n=5-6/group.
Online Figure VI: Deletion of CD36 or Nox2 in PVM ameliorates the neurovascular dysfunction induced by Aβ1-40 superfusion in WT mice. Aβ1-40 superfusion attenuates CBF responses to bradykinin, A23187 and SNAP (A-C). However, these cerebrovascular effects of Aβ1-40 are not observed in CD36→Tg and Nox2→Tg mice (A-C). The CBF response to hypercapnia is not affected in all the chimeras (D). Resting CBF and response to adenosine are not affected by Aβ superfusion or MnTBAP treatment in all groups; *p<0.05 from vehicle ANOVA and Tukey’s test; n=5-6/group.
Online Figure VII: Deletion of CD36 or Nox2 in PVM counteracts the neurovascular dysfunction in Tg2576 mice. Transplant of CD36−/− or Nox2−/− BM into Tg2576 mice rescues the attenuation in the CBF response to bradykinin (A), A23187 (B), or SNAP (C). The CBF response to hypercapnia is not affected in the chimeras. *p<0.05 from WT→WT, CD36→Tg, or Nox2→Tg; ANOVA and Tukey’s test; n=5-6/group.
Online Figure VIII: Circulating Aβ_{1-40} enters the perivascular space and reaches PVM. Vessels are visualized with DiO (red) and PVM are identified by the presence of phagocytized dextran (green). In WT mice treated with PBS, Cy5-labelled Aβ_{1-40} infused into the carotid artery (icAβ_{1-40}) is observed in the perivascular space in close association with PVM (A). In mice treated with clodronate, the signal from Cy5-labelled Aβ_{1-40} is still present in the brain, but is no longer associated with dextran-positive PVM, which are depleted (B). As a control, when vehicle is injected into the carotid artery of mice treated with PBS-liposomes (C) or clodronate (D) the Cy5 signal is not observed.
MATERIALS AND METHODS

Mice
All experimental procedures were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medicine. Experiments were performed in 3-4 month-old transgenic mice overexpressing the Swedish mutation of the amyloid precursor protein (APP) (Tg2576)1 or age-matched WT littermates, referred to as WT mice. In BM chimera experiments CD36−/− and Nox2−/− mice were used as BM donors. In some studies, GFP+ mice (JAX Stock #006567) were used as BM donors. All mice were males and derived from in house colonies2-5.

Intracerebroventricular injection of clodronate or dextran
Liposomes containing clodronate or phosphate buffered saline (PBS) were administered intracerebroventricularly (icv) as previously described6, 7. Isoflurane-anesthetized mice were placed in a stereotaxic frame. Ten μl of clodronate-liposomes (7 mg/ml) or PBS-liposomes (vehicle) were injected into the cerebral ventricles with a glass micropipette through a burr hole drilled on the right parietal bone. Mice were used in the experiments 5-7 days later, when PVM depletion is well developed and stable6, 7. In some experiments, PVM were identified by their ability to phagocytize dextran6, 8. For dextran injections, 10 µl of Alexa Fluor® 680 dextran (10,000 MW, anionic, fixable, ThermoFisher Scientific, D34680; 2.5 mg/ml) in saline or saline alone were injected and PVM labeling was examined 24 hrs later.

Labeling cortical blood vessels with DiO
Cortical blood vessels were labeled with the lipophilic dye DiO [DiOC18(3) (3,3'-Diocetylfluorescein Perchlorate)], as described9. Briefly, mice were anesthetized (5% isoflurane) and transcardially perfused with PBS (2 ml) followed by DiO (1:50, V-22886, Molecular Probes; 5ml/mouse) and then by 4% paraformaldehyde (PFA). Brains were harvested and post-fixed in 4% PFA overnight, and then cut (thickness 150 µm) using a vibratome and examined under the confocal microscope (Leica SP5).

Immunohistochemistry
Mice were anesthetized with sodium pentobarbital (120 mg/kg, i.p.) and perfused transcardially with PBS followed by 4% PFA in PBS. Brains were removed, stored overnight, and sectioned in a vibratome (section thickness: 40 µm). Free-floating brain sections were permeabilized with 0.5% Triton X-100 and non-specific binding was blocked with 1% of normal donkey serum. Sections were randomly selected and incubated with the primary antibodies CD206 (clone MR5D3, rat polyclonal, 1:200, Serotec), Glut-1 (rabbit polyclonal, 1:200, Calbiochem), Iba-1 (rabbit polyclonal, 1:500, Wako Chemicals), or GFAP (mouse monoclonal, 1:1000, Sigma) overnight at 4°C. After washing, brain sections were incubated with a Cy5- or a FITC-conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories), mounted on slides and imaged with a confocal microscope (Leica SP5). The specificity of the immunofluorescence was verified by omission of the primary and/or secondary antibody or blocking of the antigen. All quantifications were performed by investigators blinded to the treatment on randomly selected fields within the somatosensory cortex.

Identification and quantification of PVM in somatosensory cortex
PVM were identified by well established criteria, including expression of CD206, ability to phagocytize dextran and perivascular location6, 7, 10 (fig 1). The association with cortical blood vessels was confirmed by co-labeling with the endothelial marker Glut-1 (rabbit polyclonal, 1:200, Calbiochem), the smooth muscle marker α-actin or DiO9. For CD206+ PVM, randomly
selected fields (20x objective; 4 confocal images/mouse; n=5 mice/group) within the somatosensory cortex were analyzed. For dextran+ PVM, a representative coronal section from each mouse was reconstructed from tiled images taken with the confocal microscope, and the whole somatosensory cortex (n=3/group) was analyzed. ImageJ (NIH) was used for all image analyses.

**Cerebrovascular ROS measurement**

ROS production was assessed *in vivo* by hydroethidine (HE) microfluorography\(^2\,^4\,^6\,^11\). To assess ROS production in PVM, mice were first injected icv with clodronate- or PBS-liposome and 5-7 days later with dextran (see above). The day after dextran injection, in WT mice HE (2 \(\mu\)M in Ringer; Invitrogen) with or without A\(\beta\) (5 \(\mu\)M) was superfused over the somatosensory cortex. In tg2576 mice, HE (10 mg/kg) was injected into the jugular vein. BM-transplanted mice were injected with icv dextran one day before ROS measurement. Sixty minutes after HE administration, mice were injected with DiO to label cerebral blood vessels as described above. Coronal brain sections were then cut through the cortex underlying the cranial window, and ROS dependent fluorescence associated with blood vessels or PVM was quantified as described previously\(^2\,^4\,^6\,^11\).

**Bone marrow transplant**

Procedures for BM transplant have been previously described\(^6\,^12\) and are only summarized. Whole-body irradiation was performed in 7 weeks-old mice (Nordion Gammacell 40 Exactor). Eighteen hours later, mice were transplanted with BM cells (2\(\times\)10\(^6\), i.v.) isolated from the donor CD36\(^{-}\), Nox2\(^{-}\), and WT controls. Mice were housed in cages with sulfamethoxazole (0.12%; w/v) and trimethoprim (0.024%) added to drinking water for the first 2 weeks. Reconstitution of BM cells was verified 5 weeks after irradiation by testing the percentage of positive CD36 or Nox2 genomic DNA in isolated blood leukocytes\(^12\). Reference primers sequences were as follows: m:\_ICAM1\_prom.3, 5\textquoteleft-GGACTCACCTGCTGTCTCTCT-3\textquoteleft; and m:\_ICAM1\_prom.4, 5\textquoteleft-GAACGAGGGCTTCGGTATTT-3\textquoteleft; target primers sequences were as follows: CD36\_1, 5\textquoteleft- -3\textquoteleft; and CD36\_2, 5\textquoteleft- -3\textquoteleft; m:\_Cybb\_gt\_1, 5\textquoteleft-CTGCTCACGCTCTCTCCTCTA-3\textquoteleft; and m:\_Cybb\_gt\_2, 5\textquoteleft-CTGGAACCCCTGAGAAAGGAG-3\textquoteleft; (Invitrogen). qRT-PCR was conducted with 20 ng of DNA, in duplicate 15 \(\mu\)l reactions using the Maxima SYBR Green/ROX qPCR Master Mix (2\(\times\)) (Thermo Scientific). Chimerism was >95% for both CD36\(^{-}\) and Nox2\(^{-}\) BM chimeras. A PCR cycling protocol consisting of 15 s at 95°C and 1 min at 60°C for 45 cycles was used for quantification CD36 or NOX2 relative expression levels were calculated by 2 \((-\Delta\Delta \text{ CT})\) method. For studies of PVM number and distribution after BM transplant in Tg2576 mice, the BM of transgenic mice expressing GFP was transplanted into irradiated Tg2576 mice or littermates.

**General Surgical Procedures for CBF measurement**

As described in detail elsewhere\(^2\,^4\,^11\), mice were anesthetized with isoflurane (induction, 5%; surgery, 2%) and maintained with urethane (750 mg/kg; i.p.) and \(\alpha\)-chloralose (50 mg/kg; i.p.). A femoral artery was cannulated for recording of arterial pressure and collection of blood samples for blood gas analysis. The trachea was intubated and mice were artificially ventilated with a mixture of \(\text{N}_2\) and \(\text{O}_2\). Arterial blood pressure (80-90 mmHg), blood gases (pO\(_2\), 120-140 mmHg; pCO\(_2\), 30–40 mmHg; pH, 7.3-7.4), and rectal temperature (37°C) were monitored and controlled. Throughout the experiment the level of anesthesia was monitored by testing motor responses to tail pinch. The somatosensory cortex was exposed through a small craniotomy (2x2 mm). The dura was removed, and the exposed cortex was continuously bathed with a modified Ringer’s solution (36-37°C; pH: 7.3-7.4)(see ref.\(^13\) for composition). CBF was continuously monitored at the site of superfusion with a laser-Doppler probe (Vasamedic, St. Paul, MN) positioned stereotaxically on the neocortical surface and connected to a computerized data acquisition system. CBF values were expressed as percent increase relative
to the resting level. Resting CBF is reported as arbitrary laser-Doppler perfusion units (LDU). Zero values for CBF were obtained after the heart was stopped by an overdose of isoflurane at the end of the experiment.

**Experimental protocol for CBF experiments**

CBF recordings were started after arterial pressure and blood gases were in a steady state. CBF responses to whisker stimulation were recorded while gently stroking the whiskers with a cotton-tipped applicator for 60 sec. All pharmacological agents were dissolved in a modified Ringer’s solution. To test endothelium-dependent responses, the endothelial nitric oxide (eNOS)-dependent vasodilator acetylcholine (10 µM, Sigma), the Ca²⁺ ionophore A23187 (3 µM; Sigma) or bradykinin (50 µM; Sigma) was topically superfused for 3-5 min and the evoked CBF increases recorded. To test smooth muscle reactivity, CBF response to adenosine (400 µM, Sigma) or the NO donor S-Nitroso-N-acetyl-DL-penicillamine (SNAP; 50 µM, Sigma) were examined²⁴¹¹. The increase in CBF produced by hypercapnia was tested by introducing 5% CO₂ in the ventilator to increase arterial pCO₂ up to 50-60 mmHg. Once a stable increase in CBF was obtained for 3-5 min, pCO₂ was returned to normocapnia.

**Measurement of Aβ**

Aβ was measured using an ELISA-based assay, as described previously²³⁵. Briefly, the left hemispheres from the mice used for CBF studies were sonicated in 1% SDS with protease inhibitors and centrifuged. The supernatant contained SDS-soluble Aβ peptides. The pellet was sonicated in 70% formic acid and centrifuged as above. The formic acid extract was neutralized by a 1:20 dilution into 1 M Tris phosphate buffer (pH 8.0). Aβ₁₋₄₀ and Aβ₁₋₄₂ concentrations (pM) were determined in supernatant (SDS-soluble) and in the formic acid extract of the pellet (SDS-insoluble) using the BAN-50/BA-27 and BAN-50/BC005 sandwich ELISA assay.

**Statistics**

Sample size was determined by power analysis based on previous published works published by our lab on CBF regulation. Animals were randomly assigned to treatment or control group and analysis was performed in a blinded fashion. Group difference was analyzed using Student’s t test (paired or unpaired) or analysis of variance (ANOVA) with Tukey’s test, as appropriate. Data are expressed as mean±SEM and statistical difference was considered significant at p<0.05.

**References**


