Microglia constitute a barrier that prevents neurotoxic protofibrillar Aβ42 hotspots around plaques

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In Alzheimer’s disease (AD), β-amyloid (Aβ) plaques are tightly enveloped by microglia processes, but the significance of this phenomenon is unknown. Here we show that microglia constitute a barrier with profound impact on plaque composition and toxicity. Using high-resolution confocal and in vivo two-photon imaging in AD mouse models, we demonstrate that this barrier prevents outward plaque expansion and leads to compact plaque micro-regions with low Aβ42 affinity. Areas uncovered by microglia are less compact but have high Aβ42 affinity, leading to the formation of protofibrillar Aβ42 hotspots that are associated with more severe axonal dystrophy. In ageing, microglia coverage is reduced leading to enlarged protofibrillar Aβ42 hotspots and more severe neuritic dystrophy. CX3CR1 gene deletion or anti-Aβ immunotherapy causes expansion of microglia coverage and reduced neuritic dystrophy. Failure of the microglia barrier and the accumulation of neurotoxic protofibrillar Aβ hotspots may constitute novel therapeutic and clinical imaging targets for AD.

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The mechanisms by which neural circuit disruption occurs in Alzheimer’s disease (AD) are not well understood. While genetic evidence in early-onset familial AD suggests a strong involvement of abnormal β-amyloid (Aβ) processing and aggregation, in sporadic late-onset AD it is thought that disturbed Aβ clearance may lead to Aβ aggregation, neuronal injury and dysfunction. A variety of mechanisms of brain Aβ clearance have been postulated, including a role for microglia, the resident immune and phagocytic cell in the central nervous system. The involvement of these cells in AD is further supported by recent studies showing that human genetic variants in microglia-related molecules, such as TREM2 and CD33, are associated with an increased risk of late-onset AD. Microglia are highly motile cells that constantly survey the brain microenvironment and undergo activation in response to a diverse range of tissue perturbations. One striking feature of the behaviour of microglia in the AD brain is their marked clustering around fibrillar Aβ deposits, which are also in close proximity to dendrites with reduced spine density and dystrophic axons. Plaque-associated microglia display an activated and polarized morphology with their processes pointed towards and highly intertwined with the plaque surface. Despite this close interaction, in vivo mouse data have shown that microglia are very ineffective at phagocytosis of fibrillar amyloid deposits, but are instead able to take up prefibrillar forms of Aβ. In addition, modulation of microglia-related chemokine receptors or anti-Aβ immunization, both of which can affect microglia activation status, have been shown to influence the degree of brain amyloid accumulation. While these effects on amyloid burden may partly be explained by Aβ phagocytosis, microglia could possess additional unknown functions that may affect the evolution of amyloid deposition. Furthermore, due to their close proximity to axonal structures and their potential for producing neurotrophic cytokines and reactive oxygen species, some suggest that microglia play a causative role in the formation of dystrophic neurites. On the other hand, microglia could play neuroprotective roles through mechanisms not yet identified. Thus, it remains unknown whether the aspects of microglia function play beneficial or detrimental roles that could be specifically targeted for therapeutic purposes.

To address this gap in knowledge, we developed in vivo methods using two-photon and high-resolution confocal microscopy for examining the role of microglia in the dynamic equilibrium between soluble interstitial Aβ and fibrillar amyloid deposits, amyloid plaque expansion and the resulting toxicity to adjacent neurons. Our data reveal a striking pattern of anti-co-localization between microglia processes, protofibrillar Aβ and dystrophic axons. We demonstrate that this pattern is due to microglia acting as a barrier that restricts the radial expansion of plaques by controlling their affinity for soluble Aβ, a function that we show is critical for limiting the formation of neurotoxic hotspots of protofibrillar Aβ around plaques. Modulation of microglia activity by either CX3CR1 receptor deletion or passive anti-Aβ immunization leads to the expansion of the microglia barrier with a consequent reduction in plaque neurotoxicity. Finally, we show that certain natural and synthetic small molecules have the ability to selectively target these neurotoxic protofibrillar Aβ hotspots, raising the possibility that analogous compounds could be used therapeutically or in clinical imaging applications.

Results
Microglia plaque envelopment does not prevent diffusion of soluble Aβ into the plaque core. We first quantified the extent to which the surface of individual amyloid plaques was covered by the processes of adjacent microglia in two Alzheimer-like transgenic mouse models (5xFAD and CRND8). In our analysis of confocal image stacks of brain slices with labelled microglia and fibrillar amyloid plaques, we observed that larger plaques tended to have less microglia coverage than smaller ones, but overall there was a great heterogeneity in the degree of microglia coverage (Fig. 1a,b). Given that microglial processes are known to be highly motile in the normal brain, we next asked how physically stable the processes involved in tightly wrapping plaques were compared with those that did not contact plaques. To address this, we used in vivo two-photon imaging to visualize plaques and microglia in 5xFAD mice expressing green fluorescent protein (GFP) exclusively in microglia. We found that over intervals of minutes to days, the microglial processes wrapping plaques were highly stable in contrast to the motile processes in the same cells that were not contacting plaques (Fig. 1c–g and Supplementary Movie 1). However, when we monitored the same individual plaques and microglia over intervals of months, we observed some rearrangement of the microglia processes contacting plaques, with an overall significant reduction in the degree of coverage (Fig. 1e–g), consistent with our data from fixed tissues showing that the degree of microglial coverage is lower as plaques grow in size (Fig. 1b).

Given the tight and stable contact between microglia processes with the plaque surface, we asked whether this interaction was capable of restricting the binding of interstitial soluble Aβ to the plaque. We performed in vivo two-photon imaging of fibrillar amyloid plaques prelabelled with the fluorescent fibrillar amyloid-binding dye (E,E)-1-fluoro-2,5-bis(3-hydroxycarbonyl-4-hydroxy)styrylbenzene (FSB), while simultaneously microinjecting fluorescently conjugated soluble Aβ40-555 or Aβ42-555 into the subarachnoid space (Fig. 2a,b). Within minutes of injection, both Aβ40 and Aβ42 were diffused throughout the hemisphere and rapidly bound with high-affinity to pre-existing FSB-labelled fibrillar plaques (Fig. 2c–e and Supplementary Movie 2). Thus, even among the most robustly enveloped amyloid plaques, microglia processes are not capable of preventing the diffusion of Aβ into the plaque core.

Soluble Aβ42 has a high affinity for plaque microregions with low fibrillar amyloid density. We then imaged plaques at a higher resolution after subarachnoid Aβ-555 infusion and were surprised to observe a striking difference between Aβ40 and Aβ42. Aβ42-555 showed a very heterogeneous binding pattern with hotspots of markedly increased affinity (Fig. 2f), while Aβ40-555 binding was more homogeneous throughout the plaque (Fig. 2f,g, Supplementary Fig. 1a). The dramatic discrepancy in binding patterns is unlikely to be due to the differential diffusion properties of Aβ40 and Aβ42 given their similar molecular size and the fact that an identical pattern of Aβ42 hotspot binding was observed after directly exposing plaques to Aβ42 in either unfixed freshly cut or fixed and permeabilized brain slices (Fig. 3a,b). Furthermore, a scrambled Aβ42 peptide did not bind to the plaques at all (Supplementary Fig. 1c). Importantly, immunohistochemistry on fixed brain slices with C-terminal specific antibodies demonstrated that endogenous Aβ42 also accumulates in these hotspot microregions (Fig. 3c). This strongly suggests that plaque microregions have distinct biochemical conformations that cause differential and specific Aβ42 affinities leading to the observed hotspots.

We further examined the intrinsic differences between plaque microregions with or without Aβ42-555 hotspots (Fig. 4a). We found that Aβ42 hotspots invariably colocalized with regions containing low fibrillar amyloid density, as evidenced by the inverse relationship between Aβ42 and Thioflavin S (TS)
fluorescence intensities (Fig. 4b–e). Furthermore, non-hotspot areas were characterized by a more distinct TS-labelled fibrillar plaque edge, compared with the gradual tapering of TS labelling near Aβ42 hotspots (Fig. 4f–h). This indicates that the presence of pre-existing amyloid fibrils is critical for the soluble Aβ42 binding to occur, but as the amyloid plaque becomes gradually denser and compact, the affinity for new soluble Aβ42 is dramatically reduced.

Aβ42 hotspots are anti-co-localized with microglia processes. Given the incomplete plaque coverage by microglia (Fig. 1), we next explored the possibility that the patchy fibrillar plaque affinity for soluble Aβ42 is influenced by the presence of microglia processes. To examine this, we obtained high-resolution confocal images of microglia around plaques and correlated their location with the presence of Aβ42-555 hotspots and the relative density of fibrillar amyloid (TS fluorescence). We found that

**Figure 1 | Microglia process envelopment of plaques is stable over weeks in vivo.** (a) Representative confocal images of Iba1 immunolabelled microglia around Thioflavin S-labelled amyloid plaques in a 4-month-old 5xFAD mouse. (b) Quantification of microglia coverage in 4-month-old 5xFAD and 4-month-old CRND8 mice. Microglia coverage was quantified as the percentage of plaque perimeter contacted by microglia process. N = 90 plaques (1–20 μm in diameter) from five animals for each transgenic line. Red bars represent mean ± s.e.m. (unpaired t-test, t(84) = 2.998 and (156) = 2.422 in 5xFAD and t(218) = 2.379 and (362) = 2.506 in CRND8). (c) In vivo time-lapse images of the microglia around amyloid plaques in CX3CR1-GFP × 5xFAD mouse. Blue arrows: microglia processes contacting amyloid plaques; red arrows: processes not in contact with amyloid plaque and extended between 20th and 30th min of the imaging session; red arrowheads: processes not in contact with amyloid plaque and regressed between 20th and 30th min of the imaging session. (d) Quantification of microglia process stability over 30 min. N = 15 plaques in five animals. Data represent mean s.e.m. (e,f). In vivo time-lapse imaging over weeks to months reveals the stability of microglia processes contacting amyloid plaques. Stable processes: yellow arrows; newly added processes: red arrows; regressed processes: blue arrows. (g) Quantification of long-term dynamism of microglia processes contacting amyloid plaques. N = 51 plaques in nine animals. Scale bars, 5 μm in c,e and f.
plaque surface regions not covered by microglia processes had a much lower TS fluorescence intensity (Supplementary Fig. 3) but exhibited strong Aβ42-555 labelling, while immediately adjacent regions wrapped by microglia processes had greater TS fluorescence but much less Aβ42-555 labelling (Fig. 5a–c). Furthermore, smaller plaques that tended to be completely wrapped by microglia processes had minimal Aβ42 binding, while larger plaques were less covered by microglia but showed high-affinity Aβ42 binding (Fig. 5d,e).

In contrast to microglia, the location of reactive astrocytes, which also have polarized processes making extensive contact with the plaque surface, did not show any spatial correlation with TS or Aβ42-555 fluorescence (Fig. 5f and Supplementary Fig. 5). Thus, these data strongly suggest that the presence of microglial processes is a critical determinant of the degree of amyloid compaction, which markedly affects the affinity of plaques to soluble Aβ42.

Protobfibrillar Aβ42 hotspots can be revealed with specific Aβ binding dyes. Given our findings that plaque microregions with low levels of amyloid compaction in microglia-uncovered areas have a much greater affinity for exogenous Aβ42-555, we hypothesized that these areas would also be sites of active binding and polymerization of endogenously produced Aβ. However, endogenous forms of protobfibrillar Aβ are difficult to visualize in intact tissues because of a lack of specific antibodies for these Aβ conformations. Instead, we tested the properties of a variety of fluorescent small molecule dyes known to have affinity for amyloid plaques. We found that like Thioflavin S, the majority of Congo red and Thioflavin-T derivatives mainly bind to the fibrillar plaque core (Fig. 6c–h). Surprisingly, the natural product Curcumin33 and the synthetic compound THK-26534 both displayed a unique binding pattern remarkably similar to that of exogenously infused Aβ42 (Fig. 6a,b,c and Supplementary Fig. 5). Curcumin has been shown in vitro to bind oligomeric and protobfibrillar Aβ33,35. To determine whether Curcumin and THK-265 are binding to oligomeric Aβ enriched in these hotspots, we immunolabelled brain slices with the oligomer-specific antibodies A11 and OC36,37. These antibodies, however, did not show any hotspot pattern, but instead labelled the plaque perimeters uniformly as well as small puncta throughout the...
brain parenchyma (Supplementary Fig. 2), suggesting that hotspots are not enriched in Aβ oligomers. The fact that the labelling by Curcumin and THK-265 but not TS is highly co-localized with that of infused Aβ42-555 and endogenous Aβ42 immunolabelling (Fig. 3c), strongly suggests that hotspots are sites of active aggregation for endogenous Aβ42 into protofibrils.

These data demonstrate that the protofibrillar Aβ42 hotspots are determined by intrinsic biochemical differences within plaque microregions that can be unambiguously revealed in vivo by fluorescently labelled Aβ42 or specific Aβ binding dyes.

**Neuritic dystrophy is more severe next to plaque regions lacking microglia processes.** Oligomeric and protofibrillar forms of Aβ have been shown to be significantly more neurotoxic than larger Aβ aggregates. Given our finding that plaque microregions not covered by microglia, are hotspots rich in protofibrillar Aβ42, we asked whether these regions were associated with greater axonal dystrophy than the adjacent ones that were covered by microglia processes. To address this question, we first performed immunolabelling of dystrophic neurites using anti-Lamp1 or anti-Ubiquitin antibodies, which robustly demarcate the swollen dystrophic axons around fibrillar neurites (Fig. 7a–d). Quantitative confocal microscopy revealed that plaque regions covered by microglia had significantly fewer dystrophic neurites than those without microglia processes (Fig. 7b,d). We then examined the dynamics of dystrophic neurite (DN) formation and elimination by in vivo two-photon imaging and correlated it with the location of microglia processes. We used a triple transgenic strategy by crossbreeding 5xFAD mice with mice expressing GFP in microglia (CX3CR1<sup>GFP<sup>+</sup></sup>), while neurons were labelled by further crossbreeding with mice expressing yellow fluorescent protein (YFP) under the Thy1 promoter or by cortical infection with an adeno-associated virus driving neuronal expression of tdTomato. We found that areas not covered by microglia had an even greater degree of axonal dystrophy than microglia-covered areas compared with what was apparent by anti-Lamp1 or anti-Ubiquitin immunolabelling (Fig. 7e,f).

Furthermore, time-lapse in vivo imaging showed a markedly increased rate of DN formation in areas lacking microglia processes (Fig. 7g,h), supporting the concept of increased neurotoxicity by protofibrillar Aβ42 hotspots.

Taken together, these data demonstrate that plaque areas without microglia processes are sites of greater neurotoxicity likely due to the accumulation of endogenous protofibrillar Aβ42. Furthermore, it suggests that microglia act as a barrier that shields neurons from the local toxicity associated with the ongoing aggregation of Aβ.

**The neuroprotective barrier provided by microglia declines in ageing.** To explore the effects of ageing on the ability of microglia to form an effective barrier, we compared the number of microglia and their degree of coverage of the plaque surface across different ages. We found that as mice became older there was a gradual decrease in the average number of microglia within a 25-μm radius of the plaque perimeter (Fig. 8a), which was at least partly due to a diminished proliferative capacity (Fig. 8b). This reduction led to a significant difference in the degree of microglia process enrolment of the plaque surface (Fig. 8a). We then probed whether this microglia reduction had any effect on the degree of Aβ aggregation and toxicity around plaques. We first looked at the amount of protofibrillar Aβ42 by measuring the perimeter of curcumin labelling around the plaques of various ages in the 5xFAD mouse model.
sizes and at different mouse ages. Interestingly, we found that in 18 to 24 month-old CRND8 mice, plaques of equivalent size as in 4–month–old mice had a markedly increased protofibrillar A\beta42 halo (Fig. 8c,e and Supplementary Fig. 8). Furthermore, the accumulation of protofibrillar A\beta was associated with a large increase in the area of dystrophic neurites (Fig. 8d,f). These data demonstrate that microglia ageing is associated with a profound increase in the accumulation of protofibrillar A\beta around plaques and in worsened neuritic pathology, which could be partly due to an ineffective microglia barrier function.

Modulation of the microglia barrier ameliorates plaque neurotoxicity. Given our evidence of greater dystrophic neuropathology near plaque microregions lacking microglia coverage and in aged mice with fewer microglia around plaques, we hypothesized that enhancing microglia coverage would have a neuroprotective effect. We have previously shown that microglia clustering around individual plaques can be manipulated by deletion of the chemokine receptor CX3CR1 (ref. 19). We thus crossbred CRND8 with CX3CR1\textsuperscript{-/-} mice and showed that CRND8/CX3CR1\textsuperscript{-/-} mice had an increase in microglia process coverage of individual amyloid plaques (Fig. 9a,b). Furthermore, quantification of dystrophic neurites demonstrated that increased microglia process coverage in CRND8/CX3CR1\textsuperscript{-/-} mice was associated with a significant reduction in neuritic dystrophy (Fig. 9c). A similar reduction in neuritic dystrophy following CX3CR1 knockdown was observed in another A\beta amyloidosis mouse model (5 \times FAD; Supplementary Fig. 6). Interestingly,
despite the fact that microglia coverage was increased in plaques of all sizes, we only observed reduced neuritic dystrophy in relatively small plaques, while larger ones, which are likely to be older, had the same degree of dystrophy regardless of the CX3CR1 genotype (Fig. 9c).

We also tested passive anti-Aβ immunization as a method to enhance microglia process coverage given that anti-Aβ antibodies have been shown to induce microglia activation around plaques. Indeed, administration of AB9 antibody to young 5 x FAD mice over 8 weeks induced a marked increase in microglia process coverage of fibrillar plaques (Fig. 9d). Similar to mice lacking CX3CR1, the neuroprotective effect of increased microglia coverage was mainly observed in smaller plaques, which are likely to be the newest ones (Fig. 9e,f).

Taken together, we demonstrate that molecular manipulation of microglia can promote the barrier phenotype, which preferentially reduces local neurotoxicity around small early-stage plaques, suggesting that microglial processes wrapping the plaque core actively protect adjacent neurons from Aβ toxicity within a critical period during plaque development.

Figure 5 | Microglia but not astrocyte processes are anti-co-localized with Aβ42 hotspots. (a,b) Representative confocal images of Aβ42 hotspot (arrowheads) and surrounding microglia. Microglia were labelled by crossbreeding with CX3CR1-GFP mice. (b) Quantification of Aβ42-555 fluorescence in areas covered or uncovered by the microglia. N = 60 plaques from three mice. Data represents mean ± s.e.m. (unpaired t-test, t = (30)6.309).

(c-e) Microglia coverage decreases as plaques grow while Aβ42 hotspot becomes more prominent. Small plaques tend to be completely wrapped by microglia without hotspot (d). Quantification of plaque perimeters in different size groups show that plaque size negatively correlated with microglia coverage, while positively correlated with Aβ42 hotspot (e). N = 60 plaques from three mice. Data represents mean ± s.e.m; the corresponding groups in each plaque size bin were compared with unpaired t-test (t = (30)2.728 and (22)2.218 for microglia coverage, t = (30)2.253 and (22)3.223 for Aβ42 coverage).

(f,g) Astrocytic processes (labelled with GFAP (glial fibrillary acidic protein)) around amyloid plaques do not correlate with Aβ42 hotspot. N = 60 plaques from three mice. Data represents mean ± s.e.m. Scale bars, 5 μm in all panels.
Discussion

We uncovered a previously unrecognized function of microglia in AD, which could be critical in the progression of amyloid deposition and its associated neurotoxicity. Our findings provide a framework for a better understanding of the role of microglia in neurodegeneration, which has gained significant importance as a result of evidence of genetic variants in microglia-related genes.

Our data support a model in which microglia envelopment of amyloid plaques acts as a barrier that plays a critical role in modulating the degree of plaque compaction, a key factor in determining the ongoing affinity of plaques for soluble Aβ42 binding and consequent formation of protofibrillar Aβ42 hotspots. Several mechanisms could contribute to the microglia barrier function (Supplementary Fig. 9). The close physical proximity of microglia processes, which are intertwined with the growing amyloid fibrils, could act as a physical barrier that restricts their polymerization. Microglia processes create a crowded microenvironment that could prevent the normal outward elongation of the fibrils by promoting their bending or compaction. In this scenario, microglia may insulate the growing fibril tips from recruiting interstitial soluble Aβ by promoting the formation of fibril conformers with reduced Aβ affinity. The location of this physical barrier is relatively stable over months given the minimal motility of microglia process contacting the plaque compared with the highly motile processes away from the plaques (Fig. 1). Nevertheless, our fluorescent Aβ42 sequential infusion over 60 days (Supplementary Fig. 4) demonstrates a gradual shift in the hotspot location, likely due to the rearrangement of microglia processes. In addition to a physical cellular barrier, active microglia phagocytosis of oligomeric Aβ and proteolytic activity by microglial secreted enzymes could further reduce local polymerization and prevent fibril extension.

The remarkable affinity to areas not covered by microglia processes of certain Aβ-binding dyes known to bind to protofibrillar Aβ, such as Curcumin and THK-265, as well as exogenous soluble Aβ42, strongly support the concept that the microglia-uncovered areas are hotspots of active Aβ polymerization. The minimal Thioflavin-positive fibrils present in the hotspot region, is consistent with in vitro data demonstrating that low amounts of fibrils are necessary to efficiently polymerize oligomers and protofibrils from soluble monomers. The fact that these small molecular weight dyes have very different binding properties compared with similarly sized Aβ-binding dyes derived from Congo red or Thioflavin-T (Fig. 6), suggests that the high affinity for plaque areas of less compact amyloid in microglia-uncovered regions, is not due to the differences in molecular size of the ligand but rather specific biochemical affinities due to Aβ conformations. A similar phenomenon could explain the marked difference in the patterns of binding observed between Aβ40 and Aβ42.

Currently, the Food and Drug Administration approved amyloid-binding positron emission tomography (PET) tracers used in patients with suspected AD, are derived from Thioflavin-T and Stilbene, which predominantly bind to the fibrillar plaque core and have negligible affinity for the protofibrillar Aβ42 around plaques. This could explain the poor correlation observed between PET signal and the degree of cognitive dysfunction when using these compounds for clinical imaging. The observation of a markedly greater protofibrillar Aβ halo and degree of neuritic dystrophy around fibrillar plaques of equal size when comparing
young adults with ageing transgenic mice (Fig. 8) further highlights the limitations of using PET tracers with affinity for the plaque core. Our observation that the most toxic plaque areas are those that contain protofibrillar A\beta42 hotspots in microglia-uncovered regions, suggests that PET ligands with affinity to these areas could have a much greater specificity for clinical AD diagnosis and staging. Furthermore, similar molecules with specific affinity for the protofibrillar A\beta42 hotspots could be better at disrupting A\beta polymerization than those that target the plaque core and may thus have greater therapeutic potential.

Figure 7 | Microglia processes protect adjacent neurites from protofibrillar A\beta42 toxicity. (a–d) Representative confocal image analysis of the degree of microglia coverage (Iba1, green) versus extent of neuritic dystrophy (Lamp1, magenta or Ubiquitin, grey). Graphs in b,d represent the area of dystrophic neurites extending radially outward from microglia-covered or -uncovered plaque perimeter. The degree of neuritic dystrophy (y axis) is normalized by the angular plaque coverage by microglia processes (see Methods for detail). N \(>60\) plaques from three mice. Data represents mean ± s.e.m. (paired t-test, \(t=(57)4.694\) in a and \(67)4.410\) for b). (e,f) Representative confocal images of microglia and dystrophic neurites around amyloid plaques in Thy1-YFP/Cx3CR1-GFP mice. Quantification of DN number was done as in b and d. N \(=70\) plaques from four mice. Data represents mean ± s.e.m. (paired t-test, \(t=(67)8.290\)). (g,h) Two-photon transcranial in vivo time-lapse images of DN dynamics around amyloid plaques. Newly formed (white arrowhead) and stable (yellow arrowhead) dystrophic neurites (labelled in either Thy1-YFP mice or by AAV-TdT injections) were quantified (data are a combination of both labelling methods). Data comes from eight mice and 45 plaques. Data represents mean ± s.e.m. (paired t-test, \(t=(42)4.108\)). Scale bars, 5 μm in all panels.
Figure 8 | Reduced microglia coverage in ageing is associated with an increased Aβ halo and neurotoxicity. (a) Quantification of microglia coverage (black bars) and microglia number (red bars) within 25 μm from the plaque edge at different ages in CRND8 mice. N > 120 plaques from four mice per age group. Data represents mean ± s.e.m. (unpaired t-test, t = (373)3.905 and (286)5.602 in microglia coverage and t = (286)3.325 in microglia number). (b) Quantification of BrdU + (5-bromodeoxyuridine) microglia in 4 and 24 month-CRND8 mice. N = 222 and 100 field of views analysed for 24 and 4 month-old mice, respectively. Data represents mean ± s.e.m. (unpaired t-test, t = (159)4.374). (c,d) Representative confocal images of Aβ halo (labelled with curcumin in c) and neuritic dystrophy (labelled with Lamp1 immunohistochemistry in d) in CRND8 mice. Scale bars, 5 μm in all panels. (e,f) Quantification of curcumin labelled protofibrillar Aβ halo (e) and Lamp1-labelled neuritic dystrophy area (f). N = 62 plaques in e and 44 plaques in f. Data represents mean ± s.e.m. (unpaired t-test, t = (60)5.508 for e and (40)2.404 for f) mo, months.

Our findings that the overall degree of dystrophy around plaques is inversely correlated with the extent of microglia plaque envelopment, that areas covered by microglia processes have fewer dystrophic neurites and a lower rate of DN formation and that CX3CR1 knockdown and anti-Aβ immunotherapy reduce dystrophic neurites, strongly suggest that microglia exert a neuroprotective effect (Supplementary Discussion). Microglia, could act as a physical barrier that insulates plaques from their surrounding neurites, reducing their contact with potentially damaging Aβ protofibril. In addition, it has been shown in vitro that an increased ratio of Aβ42 to Aβ40 increases neurotoxicity. This is consistent with our in vivo findings of hotspots of Aβ42 that co-localize with greater neuritic dystrophy. Furthermore, the active process of Aβ polymerization by itself could be more toxic than the static presence of fibrils. Thus by creating a microenvironment that is not favourable to the ongoing Aβ42 polymerization, microglia could limit damage to the adjacent neurites.

The role of microglia is further highlighted by our findings that in ageing, the decline in microglia proliferation leads to a reduction in microglia numbers and degree of envelopment of amyloid plaques, which is associated with a dramatic increase in the size of the protofibrillar Aβ halo and in the number of dystrophic neurites. Thus, while microglia could cause secondary neurotoxicity due to their ability to secrete cytokines and reactive oxygen species, it appears that in the vicinity of amyloid plaques where they can modulate the degree of amyloid compaction, they exert a net protective effect. Our observations could provide a mechanistic explanation for the increased plaque compaction and reduced neurotoxicity recently observed after manipulating IGF-1 (insulin like growth factor 1) signalling in AD mice. It could also explain why despite equivalent fibrillar plaque load, greater oligomeric Aβ concentrations are predictive of the worse cognitive status in human patients.

Interestingly, we found that microglia envelopment was most effective at limiting protofibrillar Aβ accumulation and neuritic dystrophy around smaller plaques, even after CX3CR1 chemokine receptor deletion or anti-Aβ immunotherapy. This suggests that the importance of the microglia barrier is most prominent at the initial stages of fibrillar amyloid deposition and becomes less effective as plaques grow in size. This could explain why recent studies of microglia ablation in advanced stages of amyloidosis did not demonstrate any effect on plaque counts or neuritic dystrophy.

To what extent the microglia barrier plays a role in human AD remains to be determined. Given that in sporadic AD, the process of amyloid deposition is likely to be much slower than in transgenic models, it is possible that in human sporadic and familial AD, the microglia barrier would have unique spatial-temporal properties. Nevertheless, therapeutic interventions targeted at the microglia barrier may be most effective at the early stages of plaque evolution. However, the modest reduction...
of dystrophic neurites that we observed after anti-Aβ immunotherapy, despite a robust increase in the microglia envelopment, suggests that immunotherapy could have detrimental effects that counteract the beneficial effects of the microglia barrier. This could partly explain the failure of such therapeutic approach in clinical trials, and suggests that a more specific strategy to enhance the microglia barrier may offer more effective neuroprotection.

Together, our results demonstrate that microglia envelopment of amyloid plaques in AD is a highly coordinated process aimed at increasing the degree of compartmentalization of fibrillar amyloid deposits while limiting the accumulation of potentially neurotoxic protofibrillar Aβ aggregates and shielding neurons from them. Understanding the precise mechanisms involved in this newly identified microglial function may further elucidate their neuroprotective role in AD and other neurodegenerative disorders.

**Reagents and antibodies.** Fluorescently labelled Aβ peptides were provided by Anspec (Hyflure Fluor 555-AB42 60480-1, Hyflure Fluor 488 labelled scrambled AB42 25382, Biotin-LC-AB42 24641-01, Hyflure Fluor 488-AB40 60491-01 and Hyflure Fluor 555-AB40 60492-01). Lyophilized peptides were dissolved in dimethylsulphoxide in a concentration of 1 mg ml⁻¹ and stored as stock. Anti-Iba1 polyclonal antibody (Wako, 019-19741) was used to label microglia. Anti-GFAP polyclonal antibody (Dako, Z0334) was used to label astrocytes. Anti-Iba1 polyclonal antibody (Wako, 019-19741) was used to label microglia. Anti-Lamp1 (DSHB, 1D4B) and anti-Ubiquitin monoclonal antibody (Cell Signaling, 3936) were used to label dystrophic neurites. C-terminal specific antibodies for Aβ42 (Life technologies, 700254 and BioLegend, SIG39142-200) and Aβ40 (Millipore, AB5074P) were used for labelling amyloid plaques. 4G8 (BioLegend, SIG-39200) and MOAB-2 (Biosensis, M-1586-100), antibodies were used for labelling other Aβ fragments in the brain. Aβ conformation specific antibodies, A11 (Life Technologies, ABH0052) and OC (Millipore, AB2286), were used for labelling oligomeric Aβ. AlexaFluor-conjugated IgGs were used as secondary antibodies (Life technologies). Small molecule Aβ binding dyes used in the study are: FSB (Santa Cruz, sc-359845), Thiolavin S (Sigma-Aldrich, T1892), Thioflavin T (Sigma-Aldrich, T3516), Congo red (Sigma-Aldrich C6277), Thiazine red (MP Biochemicals, 05208297), MethoxyX (gift from W. Klunk), BTA-1 (Sigma-Aldrich, B9934), Curcumin (Sigma-Aldrich, 08511) and THK-265 (Sigma-Aldrich, R277002).

**Recombinant adeno-associated virus (AAV) production.** DNA sequence of tdTomato fluorescent protein was ligated between the inverted terminal repeat sites of an AAV packaging plasmid with CAG promoter and WPRE/SV40 sequence. HEK293T cells were co-transfected with this construct and a helper plasmid pDP2 (PlasmidFactory, #PF402) for 96 h and harvested. Virus was purified through iodixanol density centrifugation and titrated by infecting HEK293T cells.

**In vivo Aβ42 brain infusion via subarachnoid space.** Aβ peptide was injected into the mouse subarachnoid space as previously described. In brief, mice were anaesthetized with ketamine-xylazine, a thin-skull window about 1 mm in diameter was made with a dental drill at 5.0 mm anteroposterior, 2.5 mm mediolateral from bregma, A = 300 μm x 300 μm piece of skull was lifted with a 25 G needle to expose the tissue underneath, and the dura was carefully removed without touching the brain parenchyma. Aβ peptide was diluted in fresh sterile artificial cerebrospinal fluid (ACSF) (1:19 v/v) at 37°C for 10 min before placing on ice. The solution was filled into a Tygon tube connected to a polypropylene tip with an outer diameter of 70 μm. A programmable syringe pump with a Hamilton syringe was connected to the tube. The tip was then inserted into the exposed subarachnoid space and fixed with cyanoacrylate glue. 10 μl of the Aβ solution was injected at...
speed of 0.2 μl per min. After injection, the tip was removed and the mouse scalp was sutured. Mice were put on a heating pad for recovery and received Buprenex analgesia. At various time points after injection, mice were either imaged in vivo with two-photon microscopy or killed for fixed tissue confocal imaging.

**Fresh brain slice Aβ labelling.** Mice were decapitated under anaesthesia. The brains were dissociated on cooled ACSF with continuous oxygenation. After dissociation, the brains were immediately transferred onto a vibratome. Coronal sections were made at 150-μm thickness. Fluorescently labelled Aβ42 was added into the sections to make a final concentration of 2 μg/ml. Slices were incubated on a shaker for 1 h at 4 °C and then washed twice with ACSF. After the last wash, slices were fixed in 4% paraformaldehyde at room temperature for 1 h. Fixed slices were then washed with PBS before imaging.

**Fixed brain slice Aβ labelling.** Fixed brain slices were washed three times with PBS, then incubated at 95 °C for 30 min (buffer: 10 mM sodium citrate, 0.05% Tween-20 (v/v)). Slices were further washed in PBS with 0.2% Triton-X100 before adding fluorescently labelled Aβ to a final concentration of 5 μg/ml. Slices were incubated at 4 °C on a shaker for 24 h and then washed three times in PBS before mounting for imaging.

**Immunohistochemistry.** The fixed brains were sectioned to 30-μm thickness using a Leica vibratome (VT1000S). For ubiquitin, sections were incubated with an antigen retrieval buffer containing 70% formic acid in 1× PBS, then incubated at 95 °C for 2 days followed by secondary antibodies for 5 h. For amyloid plaque staining, a solution of secondary antibodies was used for a 15-min incubation before mounting for imaging.

**Quantitative analysis of Aβ42 hotspots in fixed brain slices.** Aβ labelling was visualized with a Leica SP5 laser scanning confocal microscope with a Leica 63× oil objective (NA 1.4). Images were taken with a standard step size of 0.7 μm. Emission filters of Aβ were labeled with Alexa Fluor 488 and the green channels (Aβ42-555) and the red channels (Aβ42-488) were excited at 925 nm; FSB and sulphorhodamine 101 were excited at 850 nm. To analyse Aβ binding, images were first corrected for minor movement artifacts using 'StackReg' plugin in Fiji. Subsequently different channels were merged together using the 'Merge Channels' function. Each region of interest was extracted and analyzed for intensity. Two-photon microscopy imaging was performed with a Leica TCS SP8 confocal microscope (Leica, Wetzlar, Germany) equipped with 850 nm laser. Two-photon microscopy images were used for analysis of Aβ42 binding to both live and fixed brain slices. The quantification of Aβ42 labelling was performed with ImageJ. The proportion of the plaque perimeter covered by microglia processes was calculated by summing the arcs of plaque perimeter that co-localized with microglia processes. Fluorescent intensities were normalized to a 0–1 scale with the highest pixel intensity of the image as 1. To calculate the slope of fluorescent intensity change, a linear regression was fitted to the data set 10 points before and after the thresholded plaque border. To calculate the total fluorescence in the plaque halo, all pixels within the thresholded plaque border were identified by setting the threshold for 1 s.d. above the entire image mean intensity. Five optical slices through the centre of the plaque were used for analysis. Plaques were chosen as regions with high signal intensity and with a 3 mm mediolateral from bregma. A 200 μm × 200 μm opening on the posterior side of the window was made with a 25 G needle to expose the tissue underneath. And then the dura was carefully removed. Using a motorized manipulator (Sutter instrument, MP-285), a micro-glass-pipette filled with Aβ peptide solution (10 μM, Alexa Fluor 488) was inserted into the opening. The pipette was connected with a picospritzer microinjection system (Parker Hannifin Corporation, 051-0500-900) set at 30 psi. Infusion of Aβ solution was initiated by giving several 100-ms pulses through the picospritzer.

**In vivo two-photon image of microglia dynamism.** Mice received FSB injection (intraperitoneal (i.p.) 7.5 mg/kg – 1 day before the experiment. In the experiments requiring astrocyte labelling, freshly-made sulphorhodamine 101 solution (3 mM, 100 μl per mouse) was injected (i.v.) 1 day before the experiment. Mice were anaesthetized with ketamine-xylazine, a thin-skull window about 3 mm in diameter was made at 2.0 mm anteroposterior, 3.0 mm mediolateral from bregma. The cortex was removed with a #7 tweezers (Dumont) avoiding bleeding or damage to the cortex. Topical application of thiazine red solution was used to label amyloid plaques for 30 min. A thin glass coverslip (#0) was pre-cut and fixed directly on the skull after the excess dye was washed out. In vivo two-photon microscopy imaging was performed using a Leica TCS SP8 confocal microscope (Leica, Wetzlar, Germany) equipped with 850 nm laser. Two-photon microscopy images were used for analysis of Aβ42 binding to the living brain slices. Microglia were identified by setting the threshold for 1 s.d. above the entire image mean intensity. Microglia processes were tracked by setting a threshold for 1 s.d. above the entire image mean intensity. Five optical slices through the centre of the brain were used for analysis. On each slice, the intersection between the tips of microglia process and the plasma perimeter was manually defined. The proportion of the plasma perimeter covered by microglia processes was calculated by summing the arcs of plasma perimeter that co-localized with microglia processes. DNA area was determined by setting a threshold for 1 s.d. above the entire image mean intensity. The plasma area was excluded from this area quantification. In the experiment using Thy1-YFP mice, dystrophic neurites were counted manually. To compare the area with or without microglia coverage, the coverage angle was converted to a triangular selection. The selection was then extended to the limits of the image. The overlap between this extended selection and the dystrophic area was defined as the dystrophic area in the covered region of the plaque. DNA area was normalized per angular degrees of associated plasma perimeter. That is, the DNA area was divided by the relative amount of the plaque covered or uncovered in angular degrees using the formulas below: (1) for microglia-covered areas: Dystrophy/corridor = (area of dystrophic neurites outward of microglia-covered plaque perimeter)/(degrees of arc of microglia coverage). (2) for microglia-uncovered areas: Dystrophy/ (360-arc of microglia coverage). A similar analysis was used for comparing the dystrophic neurites count, Aβ42 binding, curcumin and THK265 fluorescence. Plasma with diameters between 6 to 9 μm were used in these analyses. For each measurement, the average of the results for five optical slices was reported as a measurement for individual plaques. Statistical analyses were performed in GraphPad Prism, using two-tailed t-test assuming same s.d.

**ARTICLE**

**In vivo two-photon time-lapse imaging of Aβ binding.** Mice received FSB injection (intraperitoneal (i.p.) 7.5 mg/kg – 1 day before the experiment. In the experiments requiring astrocyte labelling, freshly-made sulphorhodamine 101 solution (3 mM, 100 μl per mouse) was injected (i.v.) 1 day before the experiment. Mice were anaesthetized with ketamine-xylazine, a thin-skull window about 2.0 mm in diameter was made at 2.0 mm anteroposterior, 3.0 mm mediolateral from bregma. A 200 μm × 200 μm opening on the posterior side of the window was made with a 25 G needle to expose the tissue underneath. And then the dura was carefully removed. Using a motorized manipulator (Sutter instrument, MP-285), a micro-glass-pipette filled with Aβ peptide solution (10 μM, Alexa Fluor 488) was inserted into the opening. The pipette was connected with a picospritzer microinjection system (Parker Hannifin Corporation, 051-0500-900) set at 30 psi. Infusion of Aβ solution was initiated by giving several 100-ms pulses through the picospritzer.

**In vivo two-photon imaging of microglia dynamism.** Mice were anaesthetized with ketamine-xylazine, a thin-skull window about 2 mm in diameter was made at 2.0 mm anteroposterior, 3.0 mm mediolateral from bregma. A 200 μm × 200 μm opening on the posterior side of the window was made with a 25 G needle to expose the tissue underneath. And then the dura was carefully removed. Using a motorized manipulator (Sutter instrument, MP-285), a micro-glass-pipette filled with Aβ peptide solution (10 μM, Alexa Fluor 488) was inserted into the opening. The pipette was connected with a picospritzer microinjection system (Parker Hannifin Corporation, 051-0500-900) set at 30 psi. Infusion of Aβ solution was initiated by giving several 100-ms pulses through the picospritzer.

**Microglia coverage and DN quantification.** Imaging conditions were the same as described above except for having three emission channels. Plaques were chosen from control image stacks. Images were numbered randomly and used for analysis. All images were processed with a customized ImageJ macro. Plaque area was identified by setting the threshold for 1 s.d. above the entire image mean intensity. Five optical slices through the centre of the plaque were used for analysis. On each slice, the intersection between the tips of microglia process and the plaque perimeter was manually defined. The proportion of the plaque perimeter covered by microglia processes was calculated by summing the arcs of plaque perimeter that co-localized with microglia processes. DNA area was determined by setting a threshold for 1 s.d. above the entire image mean intensity. The plasma area was excluded from this area quantification. In the experiment using Thy1-YFP mice, dystrophic neurites were counted manually. To compare the area with or without microglia coverage, the coverage angle was converted to a triangular selection. The selection was then extended to the limits of the image. The overlap between this extended selection and the dystrophic area was defined as the dystrophic area in the covered region of the plaque. DNA area was normalized per angular degrees of associated plasma perimeter. That is, the DNA area was divided by the relative amount of the plaque covered or uncovered in angular degrees using the formulas below: (1) for microglia-covered areas: Dystrophy/corridor = (area of dystrophic neurites outward of microglia-covered plaque perimeter)/(degrees of arc of microglia coverage). (2) for microglia-uncovered areas: Dystrophy/ (360-arc of microglia coverage). A similar analysis was used for comparing the dystrophic neurites count, Aβ42 binding, curcumin and THK265 fluorescence. Plasma with diameters between 6 to 9 μm were used in these analyses. For each measurement, the average of the results for five optical slices was reported as a measurement for individual plaques. Statistical analyses were performed in GraphPad Prism, using two-tailed t-test assuming same s.d.
the GFP and FSB channels were first merged together using the ‘Merge Channels’ function. Each region for analysis was extracted from the original XYZ stack by making a three optical slices projection centred at the middle plane of the amyloid plaque. The projected images from the same location at the different time points were aligned manually based on maximizing the overlap of the plaque centre, microglia cell bodies and lipofuscin granules. After alignment, pictures from the same location were compiled into a single stack. In each slice of the stack, the plaque regions were defined by thresholding the FSB channel (3 s.d. from the mean). The intersection between the tips of microglia process and the plaque perimeter were identified manually. The plaque perimeter was divided into 360° based on the angle from the plaque centre and coverage was calculated using the summation of areas of plaque perimeter arcs that co-localized with microglia processes. These coverage data was recorded as a 360 length binary array for each time point. In all the time series, the first imaging session was used as the reference and designated as ‘day0’. Stable processes were calculated by multiplying the array from day 0 to day 1, 5, 10 and 50. Added processes were calculated by subtracting the stable processes from day 1, 5, 10 and 50’s arrays.

In vivo two-photon imaging of DN turnover. CRND8 and 5 × FAD mice were crossed with C57SC/GRFP mice to view microglia. To label dys trophy neurites the mice were either further crossbred with Thy1-YFP mice or injected with AA-tdTomato virus through the subarachnoid space as described above. Mice were injected with FSB, i.p. (7.5 mg kg⁻¹) 1 day prior to the first imaging session to label amyloid plaques. Mice were anaesthetized with Ketamine/Xylazine. A thin craniotomy window ~500 μm in diameter was made at ~2 mm to breccia and 2.5 mm lateral to the midline. In vivo two-photon imaging was performed as described above. Amyloid plaques were identified within 150 μm of the pial surface. Individual amyloid plaques were imaged with 825 nm excitation for FSB and 950 nm excitation for both microglia (GFP) and DNs (TFP or tdTomato). Image stacks were taken for plaques at a 1-μm step size. Plaque location was recorded and each plaque was relocated 5 days later and imaged under the same conditions. For analysis, a maximum intensity Z-projection of three optical slices at the plaque centre was made. Microglia coverage was determined as described above.

DN formation and elimination events were identified based on appearance or disappearance of DNs at a second time point. Events were counted based on the angle from the plaque centre and coverage was calculated using the summation of areas of plaque perimeter arcs that co-localized with microglia processes. The probability of elimination was calculated as the number of elimination events/number of pre-existing DNs. The probability of formation was calculated as the number of events in microglia-covered or -uncovered regions/arc in degrees of microglia-covered or -uncovered plaque perimeter.

Passive immunization treatment. Ab9 administration was done as previously described22. In brief, 6-week-old 5 × FAD mice were randomly divided into three groups. One group received no injection. The second group received i.p. injection of 1 mg mL⁻¹ mouse control IgG every 2 weeks. The third group received i.p. injection of 1 mg mL⁻¹ Ab9 antibody every 2 weeks. One week after the fourth injection, mice were killed and the brains were collected for quantitative immunohistochemistry.

Statistics. Data are represented as mean ± s.e.m. Histogram plots were used to observe the approximate Gaussian distributions, upon which two-tail unpaired t-tests were employed for comparisons between the two groups. A probability of P < 0.05 was considered indicative of the significant differences between the groups. Data were additionally normalized to the average value of the measurements in the control group for better scaling of the result. In the experiments with Cx3cr1 gene knockout and Abi passivation immunization, we report statistical comparisons using both plaque numbers (Fig. 9) and animal numbers (Supplementary Fig. 7). Regardless of the method used, the results are equivalent in magnitude and statistically significant.

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Author contributions
C.C. P.Y. and J.G. designed the study. C.C., P.Y. and A.S. performed the experiments. C.C. P.Y. and J.G. analysed the data. C.C., P.Y. and J.G. prepared the manuscript. J.G. supervised the study.

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