Published in final edited form as:

Intravital. 2013 ; 2(2): . doi:10.4161/intv.25693.

Four-dimensional microglia response to anti-A β treatment in APP/PS1xCX3CR1/GFP mice

Monica Garcia-Alloza^{1,2}, Laura A. Borrelli¹, Diana H. Thyssen¹, Suzanne E. Hickman³, Joseph El Khoury³, and Brian J. Bacskai^{1,*}

¹Alzheimer Research Unit, Department of Neurology, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA USA

²Division of Physiology, School of Medicine, University of Cadiz, Cádiz, Spain

³Division of Rheumatology, Allergy, and Immunology, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA USA

Abstract

Senile plaques, mainly composed of amyloid- β (A β), are a major hallmark of Alzheimer disease (AD), and immunotherapy is a leading therapeutic approach for A β clearance. Although the ultimate mechanisms for A β clearance are not well known, characteristic microglia clusters are observed in the surround of senile plaques, and are implicated both in the elimination of A β as well as the deleterious inflammatory effects observed in AD patients after active immunization. Therefore, analyzing the direct effect of immunotherapy on microglia, using longitudinal in vivo multiphoton microscopy can provide important information regarding the role of microglia in immunotherapy. While microglia were observed to surround senile plaques, topical anti-A β antibody administration, which led to a reduction in plaque size, directed microglia toward senile plaques, and the overall size of microglia and number of processes were increased. In some cases, we observed clusters of microglia in areas of the brain that did not have detectable amyloid aggregates, but this did not predict the deposition of new plaques in the area within a week of imaging, indicating that microglia react to but do not precipitate amyloid aggregation. The long-term presence of large microglial clusters in the surrounding area of senile plaques suggests that microglia cannot effectively remove A β unless anti-A β antibody is administered. All together, these data suggest that although there is a role for microglia in A β clearance, it requires an intervention like immunotherapy to be effective.

Keywords

microglia; amyloid-beta; APP/PS1xCX3CR1/GFP mice; 10D5 antibody; multiphoton microscopy; methoxy-XO4

*Correspondence to: Brian J. Bacskai; bbacskai@partners.org.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/intravital/article/25693

Introduction

Alzheimer disease (AD) is the most common cause of dementia in the elderly and there is no known cure. Senile plaques, neurofibrillary tangles and neuronal loss are the hallmarks of AD^{1,2} and although the ultimate neurotoxic mechanisms are not completely understood, senile plaques continue to be the main therapeutic target of the disease. Senile plaques are extracellular structures mainly composed by amyloid- β (A β) peptide and both passive^{3–5} and active^{6–8} anti-A β therapies have been shown to significantly reduce senile plaque pathology and behavioral disorders in different transgenic mouse models of AD. Similarly, active immunization reduced amyloid burden^{9–11} and slowed functional decline^{11,12} in AD patients. However, these observations were counterbalanced by the development of meningoencephalitis in 6% of the treated patients in the actively immunized group. It has been suggested that these deleterious effects may be due to T-cell and microglia activation,¹³ demonstrating the need to understand both the mechanisms of clearance and the cause of the inflammatory reaction in the brain. Senile plaques are surrounded by microglia clusters both in AD patients¹⁴ and animal models^{8,15} and although microglia have been implicated in the process of A β clearance, this role remains controversial.^{6,16} Microglia activation per se does not lead to robust A β elimination,⁸ but in combination with anti-A β treatments microglia might contribute both to the effectiveness of the antibody treatment as well as the inflammatory process (for a review see refs. 17 and 18). Taking these considerations into account, studying the effect of anti-A β treatment on senile plaques and the microglial cells that surround A β deposits is still an important question to understand both AD pathogenesis and treatment. In the present study, we closely follow microglia in a transgenic mouse model of AD which develops a significant amount of senile plaques by 6 mo of age. We crossed APPswe/PS1dE9^{19,20} mice with CX3CR1-GFP mice in order to follow the microglia response to anti-A β antibody treatment in vivo and in real time, using multiphoton microscopy, and monitored the effects of immunotherapy. We found that antibody treatment led to a rapid increase in microglia density around senile plaques that resulted from increases in size and number of processes from existing microglia, but not from increased recruitment of microglia.

Results

Effect of immunotherapy on senile plaque size

Despite local recruitment of brain microglia to sites of amyloid deposition, whether resident microglia can effectively remove A β remains controversial (for a review see ref. 21), probably due to the limitation of available techniques to directly assess the role of microglia in removing senile plaques. In this sense, multiphoton microscopy offers an incomparable tool to image microglia over time in living animals during anti-A β immunotherapy treatment. Acute studies (1 h before treatment and up to 2 h after antibody application) revealed no significant effect of cranial window implantation or antibody treatment on senile plaque size, microglia burden, microglia size and number of processes either in the close proximity or far from senile plaques (Table S1). Along with this no appreciable early changes were observed in microglia process extension and retraction (Fig. S1). These acute experiments also revealed significant correlations between senile plaque size and the

microglia “burden” or density located in close proximity to the plaques (Fig. S2), suggesting that larger plaques are associated with increased microglia accumulation.

We measured senile plaque size in consecutive imaging sessions after the topical application of 10D5 antibody (1.2 mg/ml) in APP/PS1×CX3CR1/GFP mice. Reduced amyloid burden and increased microglia activation has been described in homozygous CX3CR1 mice,^{22,23} however, heterozygous mice were used along with an approach to image single senile plaques and microglial cells, regardless of the total amyloid or microglia burden in this animal model. We observed the typical microglial clusters in the immediate surround of senile plaques, as well as increased ramification of microglia in close proximity to senile plaques, supporting the role of microglia as activated agents in association with A β pathology. Some distortion was observed in blood vessels as imaging sessions progressed, probably due to cranial window implantation, and we cannot exclude that microglia were also affected by the experimental approach in the long-term. This is an inherent limitation in invasive or semi-invasive approaches to gain optical access to the brain that cannot be avoided. However, treated animals were compared with untreated animals as a control for this potential caveat.

Individual, identified plaques were monitored with longitudinal multiphoton imaging immediately after the surgery (day 1) as well as on days 2, 3, 4 and 7. Anti-A β antibody treatment showed a tendency to reduce senile plaque size as soon as 24 h after the application of the treatment. This effect reached statistical significance 3 d after the application of 10D5 and was still present on day 7, reaching ~40% reduction when compared with day 1, as previously described (Fig. 1).^{6,8} We detected a significant treatment×day effect using two-way ANOVA [$F_{(4,650)} = 2.724$; * $p = 0.029$] when 10D5 treated and untreated mice were compared. Further analysis showed a significant reduction of senile plaque size using two-way ANOVA for repeated samples followed by Tamhane test [$F_{(15,491)} = 3.906$; * $p < 0.001$, vs. day 1]. No significant differences were observed in untreated mice along the imaging sessions (two-way ANOVA treatment×day effect: [$F_{(7,137)} = 0.593$; $p = 867$]) as previously described,^{19,26,27} supporting the fact that senile plaques do not change in size at these time intervals once deposited (Fig. 1).

Long-term microglia response to antibody treatment

We analyzed the longitudinal data to assess the role of microglia in antibody mediated A β clearance. We performed consecutive imaging sessions with multiphoton microscopy to follow microglia activity immediately after the surgery (day 1) as well as on days 2, 3, 4 and 7 after 10D5 application. Apart from typical microglia clusters around senile plaques, we also observed the presence of random clusters of microglia without apparent relation to senile plaques. In order to determine whether these clusters could predict the appearance of new senile plaques, we repeatedly imaged these clusters of microglia over the course of one week. However, these clusters did not predict the appearance of a new senile plaque in this time frame, as observed in Figure 2, and in accordance with previous studies.²⁶ After 10D5 application, 50 μ m diameter circles were traced around plaques and the “microglia burden,” was determined by measuring the percentage of this area occupied by microglia. We observed that ~25% of the areas were covered by microglia, measured for each of the 5

imaging sessions in animals with plaques. CX3CR1–GFP mice treated with 10D5 as well as untreated animals were included in the study, and phantom senile plaques were used to perform parallel experiments in non-transgenic mice. In areas far from plaques or in wild-type mice, microglia occupied only ~10% of the area measured during the first imaging session. We observed a significant treatment×day effect using two-way ANOVA [$F_{(12,584)} = 3.448$; $*p < 0.001$] and further analysis using one-way ANOVA followed by Tamhane test revealed significantly higher accumulation of microglia in the close surround of the plaques in the transgenic mice when compared with wildtype mice either treated with 10D5 or untreated along the 5 imaging sessions (Fig. 3). We also observed a significant increase of microglia burden in APP/PS1 at days 4 and 7 after the surgery as a consequence of 10D5 application when compared with untreated mice: day 1 [$F_{(3,117)} = 86.402$; $*p < 0.001$ vs. treated and untreated wildtype mice], day 2 [$F_{(3,78)} = 14.921$; $*p < 0.001$ vs. treated and untreated wildtype mice], day 3 [$F_{(3,79)} = 20.989$; $*p < 0.001$ vs. treated and untreated wildtype mice], day 4 [$F_{(3,51)} = 30.119$; $*p < 0.001$ vs. rest of the groups], day 7 [$F_{(3,53)} = 99.493$; $*p < 0.001$ vs. rest of the groups] (Fig. 3). We performed similar measurements in the same areas (50 μm in diameter) located at least 25 μm from plaques in order to assess the effect of antibody application in areas with no senile plaques. Two-way ANOVA revealed no treatment×day effect when treated and untreated APP/PS1 mice were compared with treated and untreated wildtype mice (ANOVA [$F_{(12,848)} = 30.931$; $p < 0.737$]). The areas covered by microglia in transgenic mice were similar to those in wildtype mice (~25%, Fig. 3).

We next sought to determine whether this effect was due to an increase in the number of microglia around the plaques, an increase in the size of microglial cells, or an increased number of processes extending from microglia. We detected an increased number of microglial cells located in the proximity of the senile plaques in the APP/PS1 mice (day 1 [$F_{(3,126)} = 3.228$; $p = 0.025$], day 1 [$F_{(3,80)} = 3.213$; $p = 0.027$], day 3 [$F_{(3,80)} = 1.451$; $p = 0.236$], day 4 [$F_{(3,55)} = 3.885$; $p = 0.014$], day 7 [$F_{(3,33)} = 2.588$; $p = 0.07$]). However, the number of cells did not increase with time when analyzed with a two-way ANOVA for repeated samples (treatment×day effect [$F_{(12,349)} = 0.474$; $p = 0.930$]). When we counted the number of microglia cells in subfields located far from senile plaques we could not observe any differences between APP/PS1 and wildtype mice along the 5 imaging sessions [$F_{(12,248)} = 0.381$; $p = 0.968$] and the values for APP/PS1 mice were in the range of wildtype mice, supporting the focal accumulation of microglia near A β deposits (Fig. 3).

When we assessed the size of microglial cells located in the proximity of senile plaques we observed a significant treatment×day effect as a consequence of 10D5 application in APP/PS1 mice when compared with the rest of the groups ([$F_{(12,1130)} = 1.1968$; $*p = 0.024$]). Further analysis using one-way ANOVA showed that average microglia size was slightly higher in APP/PS1 mice when compared with wildtype mice (day 1 [$F_{(3,485)} = 3.925$; $*p = 0.009$ vs. treated and untreated wildtype mice]). As previously detected for the microglia burden, differences were observed in microglia size between APP/PS1 10D5 treated mice and the rest of the groups 7 d after the administration of the antibody. Whereas microglia size was slightly increased with the treatment in APP/PS1 mice, the rest of the groups showed a very stable size of individual microglia cells (day 2: [$F_{(3,117)} = 3.702$; $*p = 0.012$ vs. treated and untreated wildtype mice], day 3: [$F_{(3,207)} = 0.849$; $p = 0.469$ vs. treated and untreated wildtype mice], day 4: [$F_{(3,131)} = 3.738$; $*p = 0.013$ vs. treated and untreated

wildtype mice], day 7 [$F_{(3,96)} = 11.169$; $*p < 0.001$ vs. rest of the groups]) (Fig. 3). When we assessed the average microglia size far from senile plaques, we detected a significant treatment $\times$ day effect using two-way ANOVA [$F_{(12,3295)} = 1.886$; $p = 0.031$]. However, the increase observed in microglia size far from senile plaques did not reach the levels observed in the microglia located in the close proximity to senile plaques (Fig. 3). Moreover, further analysis using one-way ANOVA did not detect any significant treatment effect at any of the times under study (day 1 [$F_{(3,1285)} = 1.684$; $p = 0.169$] day 2 [$F_{(3,853)} = 1.796$; $p = 0.146$] day 3 [$F_{(3,606)} = 2.093$; $p = 0.1$], day 4 [$F_{(3,301)} = 0.312$; $p = 0.817$], day 7 [$F_{(3,260)} = 0.115$; $p = 0.951$]).

We also analyzed the effect of anti-A β antibody treatment on the number of processes from single microglia cells. We assessed the effect on cells located in the close proximity of senile plaques (1,963 μm^2 circles around the senile plaques). The average number of processes was ~ 4 in both the wildtype mice and the APP/PS1 mice in the first imaging sessions, however, at the end of the study, 10D5 treated APP/PS1 mice reached ~ 6 processes/cell. We detected a significant treatment $\times$ session effect using two-way ANOVA [$F_{(12,785)} = 1.789$; $*p = 0.046$] and further analysis using one-way ANOVA showed a significant increase in the number of processes in APP/PS1 mice 7 d after treatment with 10D5 (day 1 [$F_{(3,314)} = 1.608$; $p = 0.188$], day 2 [$F_{(3,155)} = 2.571$; $p = 0.056$], day 3 [$F_{(3,136)} = 1.693$; $p = 0.171$], day 4 [$F_{(3,94)} = 5.059$; $*p = 0.003$ vs. rest of the groups], day 7 [$F_{(3,97)} = 9.489$; $*p < 0.001$ vs. rest of the groups]) (Fig. 3). These data show that the sprouting process accompanies the overall increase in microglia size that occurs as soon as 3 d after 10D5 application. When we assessed the number of processes of microglial cells located at least 25 μm from the borders of senile plaques, we did not detect a treatment $\times$ day effect using two-way ANOVA [$F_{(12,2669)} = 0.621$; $p = 0.826$] (Fig. 3). An illustrative example of the long-term effect provoked by 10D5 treatment on senile plaque clearance and microglia response to the treatment is shown in Figure 4.

Discussion

At present, AD treatments are limited to the manipulation of neurotransmitter systems with acetylcholinesterase inhibitors or *N*-methyl-d-aspartate receptor (NMDAR) antagonists.^{28,29} However, other approaches are focused on blocking the production, limiting aggregation, or enhancing the clearance of A β .³⁰ In this sense, previous studies have shown the capacity of passive and active anti-A β treatments to reduce A β pathology^{5,6,8} and improve learning and memory deficits in transgenic mouse models.^{4,31,32} Human studies have also shown that active immunization with A β peptides can reduce amyloid pathology and retard memory decline in AD patients.^{11,12} However, the AN1792 clinical trial was interrupted due to the development of meningoencephalitis in 6% of the patients, and it has been suggested that the deleterious effects associated with active immunization could be triggered by T-cell and microglial activation.¹³ In order to avoid these complications, and due to the great potential of immunotherapy, new clinical trials are being performed with passive immunization using anti-A β antibodies, using approaches to minimize non-specific immune responses. Taking these considerations into account, studying the relationship between senile plaques, A β clearance, and microglial activation and dynamics has come to the forefront in evaluating potential treatments for AD. Using cranial windows and multiphoton microscopy *in vivo*, we

have been able to follow the same individual senile plaques and associated microglia longitudinally with daily imaging over 7 d in APP/PS1 mice with fluorescently labeled microglia (APP^{swe}/PS1^{dE9}xCX3CR1-GFP).

In order to determine whether cranial window implantation or 10D5 application had an acute effect on microglial cells, we performed experiments where mice were imaged for 1 h after the cranial window surgery, followed by an additional 2 h after 10D5 application. Although previous studies have suggested that thin-skull preparations have advantages over cranial windows, we determined that there was negligible microglia activation following cranial window implantation and topical antibody application in this time frame. This idea is supported by the fact that none of the parameters under study: number of microglial cells in close proximity to senile plaques, number of processes from cell body, mean microglia size or microglia burden were affected, suggesting that there is not a significant acute response to the technical approach, in consonance with previous studies.^{8,26,33,34} Moreover, intensive acute laser ablation in CX3CR1-GFP mice provoked a rapid and massive microglia response that started as soon as 2 min after the lesion that increased up to 90 min later (data not shown), demonstrating that an acute insult could lead to detectable activation of microglia. It has also been suggested that dystrophic, rather than activated microglia are associated with tau pathology in AD patients, whereas limited microglia activation can be observed in postmortem tissue associated with senile plaques.¹⁶ In our hands, microglia clusters and activation are present around every single senile plaque observed, and full branching was observed for individual microglia cells.

Long-term experiments were performed for a week after topical application of anti-A β antibody treatment (10D5). Although systemic administration of antibodies represents a more physiological approach for immunotherapy treatment, previous studies have reported similar outcomes in A β clearance, both after topical and systemic administration of anti-A β antibodies.^{6,7,35,36} The advantage of our approach is the rapid evaluation of immunotherapy treatment and the precise control of antibody concentration in the brain. We observed a significant reduction of plaque size that reached ~40% of the original size by day 7 after 10D5 application, as previously described under similar conditions.^{6,8} On the other hand, senile plaque size did not change over time in untreated mice, supporting the idea that senile plaques are remarkably stable once deposited.^{19,26} Since the same plaques were followed on a daily basis for several days after 10D5 application, and once more 7 d after the treatment, the same fields within the same animals were used for the comparison, adding new insights to previous studies.³³ It would be interesting to monitor the time course of microglial activation over longer time periods, but one week after topical application of antibody leads to dramatic effects on amyloid clearance within 3 d.^{6,8,24} Moreover, distorted neurite trajectories have also been shown to recover within this time frame.^{37,38} Antibody treatment increased the size of the microglial cells located in the proximity of senile plaques, as well as the number of cell processes. We also observed a tendency toward increased numbers of cells located in the surround of the senile plaques. Although not statistically significant, we cannot exclude the possibility that we counted microglia aggregates instead of single cells, since previous studies have described the presence of multinuclear microglia aggregates in neuroinflammatory processes.³⁹

As an overall effect, the microglia burden was increased in the areas located in the proximity of senile plaques after antibody treatment. Whether microglia recruitment around the plaques is a consequence of resident microglia activation or infiltration into the brain from the periphery is still controversial,²¹ and previous studies have shown that both mechanisms are possible.^{40,41} This effect was significantly milder in areas located further away from senile plaques or in wildtype mice, suggesting a local effect of the presence of A β and antibody treatment in the specific activation of microglia around senile plaques. Although we cannot exclude that microglia aggregates were mistakenly identified as single cells as described in previous studies,³⁹ the observed alterations occur in the local proximity of the amyloid deposits, and our data suggest that no local proliferation takes place. Similarly, although we did not specifically determine whether peripheral cells were recruited, our observations seem to indicate that microglial changes are limited and local. In our 6 month old mice, we never detected a disappearing plaque, even though extensive microglia were located in the immediate surround. This supports the idea that multiple mechanisms, both dependent and independent of microglia, likely play a role in A β plaque clearance,³³ and in our hands it appears that microglia activation per se is insufficient to remove A β in vivo.⁸ Following this idea, it has been shown that F(ab')₂ antibody fragments against A β are sufficient to lead to clearance of senile plaques, supporting the idea that direct disruption of plaques, in addition to Fc-dependent phagocytosis, is involved in the antibody-mediated clearance of amyloid- β deposits in vivo.⁶ Moreover, a recent report demonstrated that eliminating Fc-mediated microglia activation reduces neurotoxicity in cell culture experiments.⁴² However, we cannot exclude that that Fc-mediated clearance is underestimated in our study, since we did not include an IgG isotype control in our experiments, and previous studies have shown that microglial phagocytosis plays a relevant role in A β clearance.^{35,36} On the other hand, there is also evidence that with aging and disease progression microglia have a reduced ability to phagocytose and degrade A β because of reduced expression of A β phagocytic receptors and A β degrading enzymes.⁴³ Since we imaged mice at a single, older age, it is possible that the effectiveness of phagocytosis by microglia is impaired compared with younger mice, as has been previously reported.⁴³

Previous studies have shown that protofibrillar A β can be effectively phagocytosed by microglia,²³ and methoxy-XO4-positive particles⁴⁴ have been detected in microglia cytoplasm. Although we did not specifically examine protofibrillar A β microglial phagocytosis, our results are in accordance with previous studies,²³ where congophilic A β does not seem to be effectively phagocytosed by surrounding microglia. This idea is supported by the stability of senile plaque size in untreated mice, described here and in previous animal studies.^{19,26,45} A recent report confirmed this observation in human tissue samples whereby a stable distribution of senile plaque size over the course of the illness was described.⁴⁶ An additional caveat with the current work is the recent reports that the use of the CX3CR1-GFP knock in model crossed with APP mice leads to reduced amyloid deposition.^{22,23} This is unlikely to change the interpretation of our results since this would suggest the opposite result that we observed - that microglia would be more effective at clearing amyloid.

We were not able to predict the location of the appearance of a new plaque. We observed, in the course of a week, abnormal microglia clusters on rare occasions, in the absence of deposited amyloid, and we repeatedly reimaged these fields in order to determine whether these areas could predict the site of formation of a new plaque. We did not detect new senile plaques associated with the microglia clusters, suggesting that senile plaques trigger microglia cluster formation and not the other way around, as it has been suggested.^{16,26} This idea is supported by the fact that senile plaque size is correlated with microglia burden and number of microglia cells in the close proximity of senile plaques.

Together, our data support the idea that A β plaques are the triggering factor for the formation of microglia clusters and indicate that although microglia are incapable of clearing amyloid plaques on their own, they can be activated with anti-A β antibodies and may then contribute to A β clearance. The alterations in microglia morphology during immunotherapy may be related to the deleterious inflammatory effects observed in a subset of patients in the clinical trials. These results are limited to structural changes in microglial morphology. It is likely that functional alterations, for instance the release of cytokines or reactive oxygen species may contribute to the deleterious side effects of immunotherapy,⁴³ and monitoring these changes are the focus of future experiments. Ultimately, understanding the underlying mechanisms of antibody-mediated clearance and microglia activation could help in the refinement of immunotherapy treatments for AD while avoiding the inflammatory side effects.

Material and Methods

Animals

Mice harboring both the APP^{swe} and PS1^{dE9} mutant transgenes (APP^{swe}/PS1^{dE9})^{19,20,47} were obtained from Jackson Lab. This mouse was developed by co-injection into pronuclei of the two transgene constructs [mouse/human (Mo/Hu) chimeric APP695 harboring the Swedish (K594M/N595L) mutation and exon-9-deleted PS1] with a single genomic insertion site resulting in the two transgenes being transmitted as a single mendelian locus. These transgenic animals begin to deposit senile plaques by 4 mo of age and robust A β deposition can be observed by 6 mo. Green microglia^{33,48} mice have been generated by placement of GFP reporter gene into the CX3CR1 locus encoding the chemokine receptor CX3CR1, specifically expressed in microglia in the CNS in heterozygosis. CX3CR1-GFP mice were crossed with APP/PS1 mice and we used 6.5–7.5 mo old APP/PS1xCX3CR1-GFP mice in this study, in order to guarantee a significant presence of amyloid pathology,¹⁹ and aged matched CX3CR1-GFP mice. All studies were conducted with approved protocols from the Massachusetts General Hospital Animal Care and Use Committee and in compliance with NIH guidelines for the use of experimental animals.

Materials

Texas Red dextran 70,000 D was from Molecular probes, Eugene, OR. Methoxy-XO4 was a generous gift from Dr Klunk, U. Pittsburgh. 10D5 was a gift from ELAN Pharmaceuticals. Common chemical reagents were obtained from Sigma.

Surgical preparation

Surgery was performed as previously described⁴⁹ with minor modifications. The skull was removed and the dura retracted to the borders. 10D5 (1.2 mg/ml, ~40 µl) was incubated for 20 min before the cranial window was sealed with dental cement. Angiograms were performed with ~12.5 mg/ml i.v. injections of Texas Red dextran (70KD) and served as a guide to repeatedly find the same sites in the brain. Animals received methoxy-XO4 i.p. (~5 mg/Kg), a fluorescent Congo Red derivative that crosses the blood-brain barrier and binds fibrillar Aβ⁵⁰ the day before each imaging session. A wax ring was placed around the coverslip of the cortical window and filled with distilled water to create a well for the water immersion objective. Animals were imaged immediately after the window implantation (day 1) and the same fields were reimaged on days 2, 3, 4, and 7 after the surgery. A set of 3 mice were imaged for 1 h before and 2 h after the application of the antibody in order to assess the possible rapid effect of cranial window implantation and antibody treatment on microglia activation.

Multiphoton imaging and processing

As previously described⁵¹ two-photon fluorescence was generated with 800 nm excitation from a mode-locked Ti:Sapphire laser (MaiTai, Spectra-Physics) mounted on a multiphoton imaging system (Bio-Rad 1024ES, Bio-Rad). A custom-built external detector containing three photomultiplier tubes (Hamamatsu Photonics) collected emitted light in the range 380–480, 500–540 and 560–650 nm. 3-color images were acquired for plaques, microglia, and angiography simultaneously using a 20× objective (NA = 0.95, Olympus). In vivo images at low resolution (615 × 615 µm; z-step, 5 µm, depth, 200 µm approximately) were acquired to provide a map of the area, using the angiogram as a 3-D fiducial. Higher resolution images were captured to identify single neurites and plaques (125 × 125 µm; z-step, 0.8 µm, depth, 20 µm approximately). To exclude motion artifacts induced by heartbeat and breathing, image stacks were aligned using AutoDeblur software (AutoQuant). Images from the green channel (GFP microglia) were further processed with the blind 3D deconvolution function in AutoDeblur to remove background noise. 2D projections of stacks from the three channels were combined in Adobe Photoshop 7 (Adobe Systems). Low resolution stacks were used to analyze plaque size and microglia presence in the areas of interest. The size of senile plaques was analyzed by thresholding methoxy-XO4-positive regions as previously described⁵² using Image J. In order to detect possible differences between the cells included in the typical microglia clusters located in the surrounding area of senile plaques, and those located out of the “halo,” circumferential subfields 50 µm in diameter (1963 µm²) around the plaques were outlined in the green channel, showing GFP-microglia, and isolated from the background field which was colored white. Microglia appeared as bright areas that were manually thresholded so that microglia-positive regions were set black and microglia-negative were set to white. The number of black pixels was then counted using a noise reduction algorithm that counted only the number of pixels in black clusters composed of more than six contiguous pixels, as previously described.^{25,53} Using Image J, we counted the total area covered by microglia as well as the average microglia size. Fifty micrometer diameter circles were also randomly located in areas without plaques, at least 25 µm from the closest senile plaque border, and microglia burden, expressed as the percentage of area

covered by microglia, was counted in order to compare microglia distribution both close and far from senile plaques after 10D5 antibody administration.

High resolution images were used to assess the effect of antibody administration on the number of microglia cells, microglia size and the number of processes from the cell body in the proximity of the plaques as well as in areas without plaques (> 25 μ m from the closest plaque).

The same paradigms: plaque size, microglia burden, microglia size and the number of processes were analyzed in those animals where we assessed the acute effect of cranial window implantation and 10D5 application. Cranial windows were performed and selected fields were imaged before and after 10D5 application: 60 min before (session 1) and 30 min before 10D5 (session 2). Cranial windows were removed; 10D5 topically applied (1.2 mg/ml) and the same fields were immediately reimaged (session 3). Animals were reimaged 30 min (session 4), 60 min (session 5) and 120 min (session 6) after 10D application. All measurement outcomes were analyzed using Image J and Photoshop software packages at each time point.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH AG020570, EB000768 (BJB), RYC-2008-02333, ISCIII-Subdirección General de Evaluación y Fomento de la Investigación. Spain (PS09/00969), Instituto de Salud Carlos III and FEDER (European Union), cofinanced by Fondo Europeo de Desarrollo Regional “Una manera de hacer Europa” (PI12/00675), Junta de Andalucía, Proyectos de Excelencia (P11-CTS-7847) (MG-A).

Abbreviations

AD	Alzheimer disease
Aβ	amyloid-beta
GFP	green fluorescent protein

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Figure 1.

(A) Effect of anti-A β antibody (10D5) treatment on the size of individual senile plaques in APP swe/PS 1dE9xCX3CR1–GFP mice. 10D5 (1.2 mg/ml) was locally applied during cranial window implantation and individual, identified plaques were monitored with longitudinal multiphoton in vivo imaging immediately after the surgery (day 1), for the 3 next consecutive days (days 2, 3 and 4) and once more on day 7. We detected a significant treatmentXday effect using two-way ANOVA [$F_{(4,650)} = 2.724$; $*p = 0.029$] when 10D5 and control treated mice were compared. We further observed a significant animalXtreatment effect in 10D5 treated mice using two-way ANOVA for repeated samples followed by Tamhane test [$F_{(15,491)} = 3.906$; $*p < 0.001$, vs. day 1]. No significant differences were observed in untreated mice along the imaging sessions [$F_{(7,137)} = 0.593$; $p = 0.867$]. Data are representative of 25–75 plaques from 3–6 mice. (B) Illustrative example of a control mouse and the effect of 10D5 application in APP swe/PS 1dE9xCX3CR1–GFP mice on day 1 and day 7. Arrowheads point at cleared senile plaques in a 10D5 treated mouse and to new deposited plaques in control mouse. Angiograms are shown in blue (Texas red dextran 70,000 D), microglia are green and senile plaques and CAA are labeled red with methoxy-XO4 injected ip 24 h before the imaging session. Scale bar: 50 μ m.

Figure 2.

Random microglia clusters did not predict the deposition of new senile plaques when weekly (day 1 and day 7) imaging sessions were performed. Panels (**A and B**) show clusters of microglia in the absence of a dense core senile plaque, similar to those observed in the close proximity of senile plaques. Panels (**C and D**) show areas without significant clusters. In panels (**E and F**) clusters of microglia are observed in the close proximity of senile plaques. Circles mark selected cells in 50 μm selections. Scale bar: 25 μm .

Figure 3.

Effect of anti-A β antibody (10D5) treatment on microglia burden, number of microglial cells, microglia size and number of processes from cell bodies in APP swe/PS 1dE9xCX3CR1-GFP and CX3CR1-GFP mice. 10D5 (1.2 mg/ml) was locally applied during cranial window implantation and microglia were monitored with longitudinal multiphoton imaging immediately after the surgery (day1), for the next 3 consecutive days (days 2, 3, and 4) and once more on day 7. We observed a significant treatment $\times$ day effect on microglia burden using two-way ANOVA [$F_{(12,584)} = 3.448$; $*p < 0.001$] in the close proximity to senile plaques and further analysis using one-way ANOVA followed by Tamhane test revealed significantly higher areas covered by microglia in APP /PS 1 mice when compared with wildtype mice in sessions 1 and 2 and 3 ($*p < 0.05$ vs. treated and untreated wildtype mice). Along days 4 and 7 a significantly higher area was covered by microglia in the proximity of senile plaques in APP /PS 1 10D5 treated mice ($*p < 0.05$ vs. rest of the groups). No significant differences were observed between treatments in subfields located $> 25 \mu\text{m}$ from the borders of senile plaques. Data are representative of 5–140 fields from 3–6 mice. We observed a significant treatment $\times$ day effect on the number of microglia cells in the close proximity to senile plaques using two-way ANOVA [$F_{(12,691)} = 2.110$; $*p = 0.015$] and further analysis using one-way ANOVA followed by Tamhane test revealed an increase in the number of processes in 10D5 treated APP /PS 1 mice on day 7 ($*p < 0.05$ vs. rest of the groups). No significant differences were observed between treatments in subfields located $> 25 \mu\text{m}$ from the senile plaques. Data are representative of 8–548 cells from 3–6 mice. We observed a significant treatment $\times$ day on microglia size effect using two-way

ANOVA [$F_{(12,1130)} = 1.1968$; $*p = 0.024$] in the close proximity to senile plaques ($< 25 \mu\text{m}$) and further analysis using one-way ANOVA revealed that individual microglia cells were significantly bigger in APP /PS 1 mice than in wildtype mice in days 1 and 2 ($*p < 0.05$ vs. wildtype mice) and that 7 d after 10D5 treatment microglia cells were significantly larger in APP /PS 1 treated mice ($*p < 0.05$ vs. rest of the groups). No significant differences were observed between treatments in subfields located $> 25 \mu\text{m}$ from the senile plaques. Data are representative of 10–663 cells from 3–6 mice. We also detected a significant treatmentXday effect on the number of processes from cell bodies ANOVA [$F_{(12,785)} = 1.789$; $*p = 0.046$] in the close proximity of senile plaques and further analysis with one-way ANOVA showed an increase of the number of processes in APP /PS 1 10D5 treated mice on days 4 and 7 ($*p < 0.05$ vs. rest of the groups). No significant differences were observed between treatments in subfields located $> 25 \mu\text{m}$ from the senile plaques. Data are representative of 13–548 cells from 3–6 mice.

Figure 4.

Illustrative example of the effect of 10D5 application in APP swe/PS 1dE9xCX3CR1-GFP mice and CX3CR1-GFP on day 1 (immediately after 10D application), and on days 3 and 7. Insets indicate zoomed in areas in the row below where the effect of the antibody treatment on senile plaque size and microglia activation is shown in detail. Arrowheads point at cleared senile plaques in an APP /PS 1 10D5 treated mouse. Angiograms are shown in blue (Texas red dextran 70,000 D), microglia are green and senile plaques and CAA are red

following methoxy-XO4 labeling 24 h before the imaging session. Scale bar: 50 μm in original images and 12 μm in zoomed areas.