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Young microglia restore amyloid plaque clearance of aged microglia

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 30 May 2016

Thank you for sending your manuscript to The EMBO Journal. Three referees have now seen your study and their comments are provided below.

As you can see the referees find the analysis interesting and insightful. However, they also raise some issues that should be resolved to make the analysis more conclusive. One major issue raised is that the developed slice system needs to be better characterized. You might already have some of the requested data on hand to address the questions related to this aspect. Western blot or Elisa is also needed to quantify Abeta levels. Referee #3 also asks for data on the effects of blocking GMCSF or its receptor to see how important this system is for amyloid clearance.

Should you be able to address the raised concerns then I would like to invite a revised version. I should add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to resolve the major concerns at this stage. Let me know if we need to discuss anything further.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The current manuscript by Daria and co-workers provides evidence that the lost ability of aged AD microglia to remove A beta plaques can be restored by young microglia. As responsible soluble factor GM-CSF has been identified in this study. Moreover, the authors describe a novel tissue culture system that allows to study Ab plaque removal by microglia in an in vitro setting. This is an interesting and timely report. It is known for some time now that microglia in the AD show reduced (or lost) Ab uptake capacities and the current manuscript may thus point towards novel strategies in AD treatment.

Recommendations

1) Microglia are shaped by their environment. To culture OHSCs from old animals is extremely challenging as usually the tissue (or the neurons in it) does not survive long. Fig. 1A shows a young slice, which looks good but an old one is not shown. How many neurons are alive in the old OHSCs? What is the morphology of microglia away from Ab plaques? Do these cells gain a ramified morphology after 14 days in culture, as it usually is the case in slices from young animals? This information is crucial for the interpretation of the data. In case tissue integrity may not be "healthy" this could potentially influence old microglia and make them responsive to GM-CSF as shown here.

2) Mostly microglia are shown with CD68, which makes it difficult to see single cells or the morphology. Alternative microglia markers like Iba1 should also be used.

3) The data on CD68, Abeta plaques etc are mostly based on the quantification of the immunosignal. Those data should be controlled by other methods, for example Western Blotting.

4) How can the CD68 staining in Fig. 4A be explained? If all of the microglia are removed by Clo treatment, that staining should also be undetectable. Yet there is almost 40% of the CD68 signal left. Could this be due to infiltration of the tissue by young microglia? Fig. EV3 on this issue is not of very high quality. Why is there green signal mostly at the edge between the tissues and very little in the young tissue? How far are the young microglia migrating into the old tissue?

5) Figure 7, 8 and 9 are suggestive with respect to the CD68 staining. It is hard seeing the described differences. Another quantification method (Western Blotting) should be provided.

Referee #2:

In this paper the authors describe an in vitro system to investigate amyloid plaque clearance by so-called young or old microglia. The co-culture system is indeed novel as it involves slice cultures from neonatal wild-type mice and 10-20 month old adult APPPSI mice, a transgenic line with a significant load of amyloid. A major problem in the interpretation of these experiments is that the authors have not characterized the novel in vitro adult tissue slices in any detail.

The reader has no idea of the constituents or integrity of a 7-14DIV slice from an adult 10-20month old APPPS1 mouse. I would be very surprised if any neurons retain their physiological integrity by this time point and presumably the majority will have degenerated by 7DIV. In the Methods it is noted that PI is used to stain for dying cells but there is no characterisation of the cell death that must occur at some stage during the slice incubation. The only neuronal staining is a NeuN field (EV2) from a young WT animal and we are asked to assume that clodronate has no impact on neuronal survival. There is no image to compare this with, so it is not helpful, and I do think that simply looking at a NeuN stained slice will inform on the amount of cell loss, neonatal or adult. (As an aside: the impact of free clodronate in vitro is a very different matter from the in vivo situation

where it is rapidly cleared. Somewhat lower concentrations than those used here have a rapid and significant impact on astrocyte survival in vitro (Kumamaru et al 2012)). Do these lower concentrations impact astrocyte function? I also assume there will be significant glial reaction -from both astrocytes and microglia in an adult APPPS1 slice - and both populations will be reactive. The microglia in the slices illustrated have undergone a dramatic morphological change relative to what is seen in vivo and their relationship to the plaque cores is also very different to that seen in vivo. There is now a body of evidence demonstrating that microglia in vitro bear little phenotypic resemblance to the cells in vivo (Butovsky et al) and this needs to be taken into account.

In addition to the failure of the authors to characterize the in vitro slice model the experimental design seems flawed. Why have the authors compared only young wild-type mice and aged 10-20mo APPPS1 mice? To study the impact of ageing of phagocytic cells in the APPPS1 mice surely there needs to be a young APPPS1 control group and an aged wild-type group. In the absence of these two groups perhaps all of the results could be simply explained by the fact that there is a difference in slices, and the microglia, derived from wild-type and transgenic animals. The lack of measurement of soluble amyloid-beta components is also a major weakness. The fact that the fibrils can not be seen does not mean they are not in solution in the petri-dish.

I accept that the authors have done an enormous amount of work and have observed some potentially interesting phenomena but their relevance to the mechanisms of amyloid clearance, or its failure, in the ageing brain are by no means clear.

Referee #3:

Daria et al pose the question whether microglia have a role in amyloid clearance in the brain and whether old microglia loose this capacity. To address this question and to determine if the potential defect could be rescued they develop an ingenious organotypic slice culture system in which they co-culture brain slices from old mice with those from young mice. They report that young brain slices induce the clearance of diffuse amyloid around plaques in old slices and that is dependent on the presence and proliferation of microglia in old slices, and that young microglia are the source of activities that trigger amyloid clearance. By testing a handful of protein factors, they find GMCSF to promote the clearance of amyloid. Overall, the system is intriguing and potentially useful for the identification of factors that promote amyloid clearance. However, the study would benefit from a few additional controls and validation studies.

1. All findings rest on histochemical analysis of plaque or microglia stains. Abeta levels should be quantified in slices using ELISA or western blot. This would, ideally, include measurements of total Abeta and Abeta42.

2. Microglial ablation should be quantified (e.g. number of microglia remaining) and related to the clearance effect. The interpretation of the findings is also somewhat questionable. Chlodronate will leave large numbers of dead cells behind which may be cleared by astrocytes and will likely trigger a strong injury response. If astrocytes or other mechanisms are necessary or contributing to amyloid clearance, these effectors may not work sufficiently anymore.

3. GMCSF or its receptor should be blocked to get a sense of how important this factor is in the observed effects. Since no unbiased method was used to discover GMCSF it is difficult to estimate the importance of this factors. Granted, the authors show sufficiency of GMCSF to induce amyloid clearance, but this is known already (e.g. Boyd and Potter, 2010)

Minor:
- CD68 may not necessarily be a marker of phagocytosis (page 5) but a more general marker of microglial reactivity

1st Revision - authors' response 19 October 2016

Reviewer #1

Comment 1: *Microglia are shaped by their environment. To culture OHSCs from old animals is extremely challenging as usually the tissue (or the neurons in it) does not survive long. Fig. 1A shows a young slice, which looks good but an old one is not shown. How many neurons are alive in the old OHSCs? What is the morphology of microglia away from Ab plaques? Do these cells gain a ramified morphology after 14 days in culture, as it usually is the case in slices from young animals? This information is crucial for the interpretation of the data. In case tissue integrity may not be "healthy" this could potentially influence old microglia and make them responsive to GM-CSF as shown here.*

Changes/Reply 1: The reviewer raised an important point. In contrast to young and neonatal OHCSs–where neuronal viability remains high–neurons from old OHSCs cannot be maintained viable. We now provide analyses of cell viability and integrity in young and old brain slices (new Fig 1B-F and Fig EV1) to clarify this issue. In the old tissue, we detected approximately 70% reduction in cell viability already at 7 DIV and more than 80% reduction in cell viability at 14 DIV (Fig 1B and C), as revealed by quantifications of propidium iodide (PI) positive cells. Cell viability of young and old tissue in co-cultures was not different compared to single cultures (Fig 1C). Cellular marker analysis revealed reduced numbers of neurons (visualized by NeuN) and astrocytes (detected by GFAP) already at 7 (Fig 1D and E), and furthermore at 14 DIV (Fig EV1A and B). As a control, we showed that integrity of neurons and astrocytes is not compromised in freshly cut (0 DIV) APPPS1 slices (Fig for reviewer's assessment only; Fig RA1). Western blot analysis confirmed reduced levels of neuronal (β3-Tubulin; Tuj1) and astrocytic (GFAP) markers in the old tissue at 14 DIV (Fig EV1D). In contrast, we detected an increased level of microglial marker CD68 in old brain slices (Fig 1F and Fig EV1C and D), particularly upon co-culturing with young slices. In summary, as reported by others (Mewes et al, 2012; Staal et al, 2011), the viability of old neurons/astrocytes in OHSC is poor also in our hands, but, surprisingly, we show here that microglia can be maintained in such environment and more importantly can re-gain their phagocytic function. This issue is also discussed in our response to Reviewer #2, Changes/Reply 1. We apologize for not clarifying this issue in the original manuscript. We believe that it is important to study microglial response to both amyloid plaques and neurodegeneration. Transgenic mouse models, such as APPPS1 used here, may be limited for studies of microglial responses *in vivo* due to the fact that no overt neurodegeneration has been observed (Ashe & Zahs, 2010). Microglial responses in human AD brains will likely also be influenced by ongoing neurodegeneration. However, our results pinpoint that even in the presence of dying neurons/astrocytes microglial phagocytic capacity can be restored and this may be of relevance for therapeutic approaches. We discussed this issue on the pages 12/13 of the revised manuscript.

As pointed out by the reviewer, we also analyzed microglial morphology in the revised version of the manuscript (new Fig 5D and E). APPPS1 mouse brains at stages used for our analysis (10-20 months) exhibit high amyloid plaque load. Thus, we could not assess microglial morphology away from plaques, but included into our analysis microglial cells not directly in contact with plaques. We compared old microglial cells at 10 DIV (cultured in the absence and presence of young slices) with microglia from freshly cut (0 DIV) APPPS1 brain slices to take ageing factor and plaque load into account. Culturing of old APPPS1 brain slices alone already changed microglial morphology from ramified to amoeboid (Fig 5D and E). This can likely be attributed to increased cell death observed in the old tissue (Fig 1B and C) because only a mild increase in number of amoeboid cells has been observed upon culturing of young tissue in the absence of old tissue. Importantly, the amoeboid morphology of old APPPS1 microglial cells is potentiated by the presence of young tissue (Fig 5D and E) and well in line with increased phagocytosis observed upon co-culturing of old and young brain slices.

Comment 2: *Mostly microglia are shown with CD68, which makes it difficult to see single cells or the morphology. Alternative microglia markers like Iba1 should also be used.*

Changes/Reply 2: As discussed above, we included analysis of microglial morphology using the CX3CR1+/GFP reporter mouse strain in the revised version of the manuscript (new Fig 5D and E). It has been demonstrated that microglial morphology elucidated by CX3CR1 is comparable to results obtained using microglial marker Iba1 (Baron et al, 2014). Microglial morphology is furthermore presented in Figures EV3 and EV4 of the revised manuscript and in Figures for reviewer's assessment only; RA3 and RA4.

Comment 3: *The data on CD68, Abeta plaques etc are mostly based on the quantification of the immunosignal. Those data should be controlled by other methods, for example Western Blotting.*

Changes/Reply 3: Protein levels of various cellular markers and Aβ upon co-culturing of old and young brain slices have been analyzed by Western blotting and included in the revised version of the manuscript (Fig EV1D and Fig 2C and D). This new data are fully in line with our major conclusions namely that increased number/activity of microglial cells is accompanied by increased levels of CD68 and increased phagocytosis of amyloid plaques is reflected by reduced levels of Aβ upon co-culturing of old and young brain slices. Of note, Western blot analysis of brain slices was feasible only on formic acid extractable aggregated Aβ as already reported by others (Humpel, 2015) while our immunofluorescence analysis focused on clearance of the plaque halo. Nevertheless, although we could readily detect a strong reduction of aggregated Aβ upon co-culturing (Fig 2C and D), this was not the case upon addition of GM-CSF to old slices (Fig for reviewer's assessment only; RA2A). This suggests more robust stimulation of Aβ phagocytosis in the co-culture model compared to GM-CSF addition as diffuse Aβ (found at the plaque halo) can be cleared more effectively compared to fibrillar Aβ deposits (plaque core) (Mandrekar et al, 2009; Nicoll et al, 2006; Serrano-Pozo et al, 2010). In agreement with these results, we also observed a stronger CD68 upregulation by Western blot analysis of co-cultures compared to GM-CSF addition (Fig for reviewer's assessment only; RA2B). This result is consistent with our conclusion that GM-CSF is not the (only) factor necessary for modulating plaque phagocytosis in the co-culture model and is also further supported by our analysis of GM-CSF knockout (GM-CSF-/-) mice (Fig 8D-F, please also refer to our response to Reviewer #3, Changes/Reply 3).

Comment 4: *How can the CD68 staining in Fig. 4A be explained? If all of the microglia are removed by Clo treatment, that staining should also be undetectable. Yet there is almost 40% of the CD68 signal left. Could this be due to infiltration of the tissue by young microglia? Fig. EV3 on this issue is not of very high quality. Why is there green signal mostly at the edge between the tissues and very little in the young tissue? How far are the young microglia migrating into the old tissue?*

Changes/Reply 4: In order to address the reviewer's concern, we analyzed possible infiltration of young microglia after clodronate treatment of old brain slices by using the microglial reporter line CX3CR1+/GFP. First, old APPPS1 brain slices (GFPnegative) were treated with clodronate or vehicle control and then cocultured with young CX3CR1+/GFP brain slices. We could detect sparse GFPlabeled young microglial cells migrating towards the old tissue (Fig for reviewer's assessment only; Fig RA3A), but did not detect any GFP-labeled cells inside of the old tissue (Fig for reviewer's assessment only; Fig RA3B). These new data are consistent with analysis of microglial migration under our standard co-culture conditions (former Fig EV3; now replaced with higher

resolution images and shown as new Fig EV4). Although under co-culture conditions we also observed migration of young microglial cells towards the old tissue, we could only detect a few GFP-labeled microglial cells in the old tissue at the direct proximity to the young tissue (Fig EV4A, arrowhead), but none inside of the old tissue (Fig EV4B). These results suggested that the remaining CD68 signal (current Fig 4C and D) questioned by the reviewer corresponds to old microglial cells and implies that clodronate treatment of old brain slices is less efficient compared to treatment of young brain slices (Fig EV3A and B). This result prompted us to quantify the efficiency of clodronate treatment in old APPPS1/CX3CR1+/GFP and young CX3CR1+/GFP brain slices. Depletion efficiency upon clodronate treatment of old brain slices was approximately 65%, as revealed by quantification analysis using two different microglial markers, CX3CR1 and CD68 (Fig for reviewer's assessment only; Fig RA4). This is in agreement with almost 40% of the CD68 signal left after clodronate treatment of old brain slices and their subsequent co-culturing with young slices (Fig 4D). In contrast, clodronate treatment of young brain slices resulted in the complete removal of microglial cells (Fig EV3).

Comment 5: *Figure 7, 8 and 9 are suggestive with respect to the CD68 staining. It is hard seeing the described differences. Another quantification method (Western Blotting) should be provided.*

Changes/Reply 5: As indicated above (Changes/Reply 3), we could detect by Western blot analysis an increased level of CD68 upon co-culturing of old tissue together with the young, as well as upon GM-CSF addition (albeit to a lower degree), supporting our conclusions based on immunoblotting. Data are presented in Figure EV1D and Figure for reviewer's assessment only; RA2B.

Reviewer #2

Comment 1: *A major problem in the interpretation of these experiments is that the authors have not characterized the novel in vitro adult tissue slices in any detail. The reader has no idea of the constituents or integrity of a 7-14DIV slice from an adult 10-20month old APPPS1 mouse. I would be very surprised if any neurons retain their physiological integrity by this time point and presumably the majority will have degenerated by 7DIV. In the Methods it is noted that PI is used to stain for dying cells but there is no characterisation of the cell death that must occur at some stage during the slice incubation.*

Changes/Reply 1: We have included an in depth characterization of the newly established *ex vivo* model in the revised version of our manuscript (new Fig 1B-F and Fig EV1). We quantified cell death using PI and performed detailed analysis (immunofluorescence at 0, 7 and 14 DIV and Western blotting at 14 DIV) of corresponding cellular markers for neurons, astrocytes and microglia. In the old tissue, we detected approximately 70% reduction in cell viability at 7 DIV and more than 80% reduction at 14 DIV (Fig 1B and C). Correspondingly, cellular marker analysis demonstrate reduced numbers of neurons (visualized by NeuN) and astrocytes (detected by GFAP) and increased number/activity of microglia (detected by CD68) already at 7, and furthermore at 14 DIV (Fig 1D-F and Fig EV1A-C). Western blot analysis confirmed reduced levels of neuronal (β3-Tubulin; Tuj1) and astrocytic (GFAP) markers and increased levels of microglial marker (CD68) in the old tissue at 14 DIV (Fig EV1D). Please, also refer to our response to Reviewer #1, Changes/Reply 1 where we discussed this issue in more depth.

Comment 2: *The only neuronal staining is a NeuN field (EV2) from a young WT animal and we are asked to assume that clodronate has no impact on neuronal survival. There is no image to compare this with, so it is not helpful, and I do think that simply looking at a NeuN stained slice will inform on the amount of cell loss,*

neonatal or adult. (As an aside: the impact of free clodronate in vitro is a very different matter from the in vivo situation where it is rapidly cleared. Somewhat lower concentrations than those used here have a rapid and significant impact on astrocyte survival in vitro (Kumamaru et al 2012)). Do these lower concentrations impact astrocyte function? I also assume there will be significant glial reaction -from both astrocytes and microglia in an adult APPPS1 slice - and both populations will be reactive.

Changes/Reply 2: Despite several publications showing that clodronate action in brain slices is directed towards eliminating microglia (Hellwig et al, 2015; Ji et al, 2013; Kohl et al, 2003; Kreutz et al, 2009; Vinet et al, 2012), we agree with the reviewer that clodronate treatment may directly and/or indirectly influence other cells such as astrocytes or neurons. Due to the fact that old astrocytes, like neurons, do not survive *ex vivo*, we omitted their analysis after clodronate treatment in the original version of the manuscript. This additional control analysis is now provided (Fig for reviewer's assessment only; Fig RA4A and B) and supports our conclusion that microglial depletion (as neuronal and astrocytic cell death will occur independently and to the same extent in both vehicle control and clodronate-treated slices) is responsible for better preservation of amyloid plaque morphology. Accordingly, we now show that in both vehicle control and clodronate-treated slices we detected no GFAP immunopositivity and only sparse NeuN immunoreactivity, reflecting poor viability of neurons and astrocytes (Fig for reviewer's assessment only; Fig RA4A and B). Additionally, as questioned by the reviewer, we tested clodronate concentrations used in our experiments (100 µg/ml) for their effect on astrocyte survival *in vitro*. In contrast to 20-fold higher concentrations used in Kumamaru *et al.* (2012) that can cause toxicity, we could not detect toxicity upon treatment of primary mouse astrocytes with 100 µg/ml of clodronate. Of note, clodronate concentration of 1000 µg/ml caused toxicity also in our experimental setup (Fig for reviewer's assessment only; Fig RA5).

Finally, the importance of old microglial cells for plaque phagocytosis in our *ex vivo* model is further proven by culturing of old brain slices with the supernatant from neonatal microglial cells (Fig 6C). We could therefore–also independent of clodronate treatment–show that old microglial cells phagocytose amyloid and that their phagocytic capacity can be enhanced by factors secreted from young microglia.

Comment 3: *The microglia in the slices illustrated have undergone a dramatic morphological change relative to what is seen in vivo and their relationship to the plaque cores is also very different to that seen in vivo. There is now a body of evidence demonstrating that microglia in vitro bear little phenotypic resemblance to the cells in vivo (Butovsky et al) and this needs to be taken into account.*

Changes/Reply 3: We fully agree with the reviewer that microglial analysis *in vitro* is fairly complicated due to differences being observed in microglial signature in cultured cells *vs in vivo*. However, culturing young (neonatal) organotypic brain slices offers benefit to formerly analyzed cultured primary microglia (Butovsky et al, 2014) due to the fact that microglia in slice cultures are surrounded by neurons and astrocytes and therefore in their more natural *in vivo*-like environment. We compared morphological properties of young microglial cells from freshly cut brain slices (0 DIV) with microglia cultured *ex vivo* (10 DIV) and could observe only a minor increase in number of amoeboid cells (Fig 5D and E) and ramification index (Fig for reviewer's assessment only; Fig RA6) upon culturing. As pointed out by others (Masuch et al, 2016), microglia *ex vivo* morphologically fully resemble those *in vivo* which is not the case with primary microglia cultured in the absence of neurons and astrocytes. However, it would be of interest to perform similar microglial signature analysis and compare molecular properties of microglial cells ex *vivo*

with those *in vivo*. Such analyses would be informative, but are beyond the scope of our manuscript.

In order to address the reviewer's concern regarding morphological changes in the *ex vivo* co-culture system described here, we have analyzed in more detail microglial morphology in old and young brain slices under following conditions: 1) freshly cut (0 DIV), 2) cultured alone for 10 DIV and 3) in coculture for 10 DIV (new Fig 5D and E). As observed by the reviewer, old APPPS1 microglial cells undergo morphological changes already when maintained alone (likely due to neurodegeneration) and particularly upon coculturing with young microglial cells. Ramified microglial morphology typically observed in young brain slices is rarely observed in old microglial cells *ex vivo* of which approximately 70% show amoeboid morphology when cultured alone and approximately 90% when co-cultured with young brain slices (Fig 5E). The described morphological changes fully correlate with differences in activation and phagocytic capacity of APPPS1 microglial cells *ex vivo*. Although APPPS1 microglia were also shown *in vivo* to display an activated phenotype, including enlargement of the soma and amoeboid appearance, their activation seem to be reduced in the later stages of plaque pathology (Scheffler et al, 2011). This is in agreement with failure of APPPS1 microglia to phagocytose amyloid (Krabbe et al, 2013) and may explain morphological differences pointed out by the reviewer.

Comment 4: *In addition to the failure of the authors to characterize the in vitro slice model the experimental design seems flawed. Why have the authors compared only young wild-type mice and aged 10-20mo APPPS1 mice? To study the impact of ageing of phagocytic cells in the APPPS1 mice surely there needs to be a young APPPS1 control group and an aged wild-type group. In the absence of these two groups perhaps all of the results could be simply explained by the fact that there is a difference in slices, and the microglia, derived from wild-type and transgenic animals.*

Changes/Reply 4: According to reviewer´s recommendations, we have included other control groups into the revised version of the manuscript (new Fig EV2D). Independently of whether we use young WT or APPPS1 brain slices, we observe similar beneficial effect on clearance of amyloid plaque halo. This is in line with published data suggesting no phagocitic defects in microglia from young AD mouse model (Hellwig et al, 2015). In addition, we also co-cultured aged WT brain slices together with aged APPPS1 brain slices and observed no significant effect on clearance of amyloid plaque halo (Fig EV2D). Furthermore, as already presented in the original manuscript, we also tested supernatants of aged APPPS1 brain slices for their effects on stimulation of plaque phagocytosis of old APPPS1 microglial cells and observed no benefical effect (Fig 6A). Taken together, our data suggest that microglial age, rather than genotype, is instrumental for the observed differences in plaque phagocytosis.

Comment 5: *The lack of measurement of soluble amyloid-beta components is also a major weakness. The fact that the fibrils can not be seen does not mean they are not in solution in the petri-dish*.

Changes/Reply 5 The fact that co-culturing of old and young brain slices or GM-CSF application are necessary to stimulate amyloid plaque clearance argues against a diffusion of amyloid from the tissue into the media. Furthermore, by blocking phagocytosis with cytochalasin D we could inhibit clearance of the plaque halo, further supporting active uptake via phagocytosis (Fig 3F and G). Another level of evidence supporting microglial phagocytosis rather than passive diffusion is the fact that we could detect Aβ within intracellular compartments of microglial cells upon co-culturing of young and old brain slices (Fig 5C). However, we have no evidence that all Aβ ingested by microglial cells is indeed degraded and that there is no release of fibrillar

Aβ into the media. Unfortunately, our *ex vivo* culturing conditions include media exchange every 3-4 days and therefore do not allow sufficient accumulation of soluble Aβ to be detected, as already reported (Humpel, 2015). Neverthless, in the revised version of our manuscript we provide further biochemical evidence for the reduction of formic acid extractable aggregated Aβ upon co-culturing of old and young brain slices (Fig 2C and D) that is fully in line with increased phagocytosis.

Reviewer #3

Comment 1: *All findings rest on histochemical analysis of plaque or microglia stains. Abeta levels should be quantified in slices using ELISA or western blot. This would, ideally, include measurements of total Abeta and Abeta42.*

Changes/Reply 1: Western blot and quantification analysis revealed decreased levels of Aβ upon co-culturing of old brain slices together with young (Fig 2C and D). This correlated with increased protein levels of CD68 (Fig EV1D). Moreover, as questioned by the reviewer, we could show that decreased levels of total Aβ resulted from reduction in levels of both Aβ40 and Aβ42 (Fig for reviewer's assessment only; Fig RA7). As this issue has also been raised by Reviewer #1, please refer to our response to Reviewer #1, Changes/Reply 3 where we discussed our new analysis in more depth.

Comment 2: *Microglial ablation should be quantified (e.g. number of microglia remaining) and related to the clearance effect. The interpretation of the findings is also somewhat questionable. Chlodronate will leave large numbers of dead cells behind which may be cleared by astrocytes and will likely trigger a strong injury response. If astrocytes or other mechanisms are necessary or contributing to amyloid clearance, these effectors may not work sufficiently anymore.*

Changes/Reply 2: We quantified microglial ablation upon clodronate treatment in the revised version of our manuscript using the CX3CR1+/GFP reporter mice. Depletion efficiency upon clodronate treatment of old brain slices was approximately 65%, as revealed by quantification analysis using two different microglial markers, CX3CR1 and CD68 (Fig for reviewer's assessment only; Fig RA4). We detected a more robust and almost complete depletion of young microglial cells (Fig EV3) upon clodronate treatment. Due to successful depletion of young microglial cells, amyloid plaque clearance in the co-culture (where young brain slices were pre-treated with clodronate, Fig 4B) was similar to baseline observed upon culturing of old tissue alone (Fig 6A, white bar at 14 DIV). Despite lower depletion efficiency, clodronate treatment of old microglial cells and subsequent co-culturing with young brain slices reduced amyloid plaque clearance even further. This can be explained by the fact that old microglial cells are the Aβ engulfing cells in our model (Fig 5B and C) and therefore their reduction has even more profound effects on amyloid plaque clearance.

Although, we could not detect overt changes in numbers of neurons or GFAP activity after clodronate treatment of young brain slices (Fig EV3A and B), we agree with the reviewer that clodronate treatment may directly and/or indirectly influence other cells such as astrocytes or neurons at the molecular level. Interpretation of clodronate treatment in the old tissue is indeed more complicated as we know that degeneration of neurons and astrocytes occurs in our *ex vivo* model system (Fig 1D and E) and the same is the case also after clodronate treatment (Fig for reviewer's assessment only; Fig RA4A and B). Unfortunately, we can therefore not assess contribution of other cells than microglia to amyloid clearance. This issue is further discussed also in our response to Reviewer #2, Changes/Reply 2.

Comment 3: *GMCSF or its receptor should be blocked to get a sense of how important this factor is in the observed effects. Since no unbiased method was used to discover GMCSF it is difficult to estimate the importance of this factors. Granted, the authors show sufficiency of GMCSF to induce amyloid clearance, but this is known already (e.g. Boyd and Potter, 2010)*

Changes/Reply 3: We fully agree with the reviewer that although our data presented in the original manuscript showed that GM-CSF addition to the old tissue is sufficient to induce amyloid clearance and are therefore in line with the published data (Boyd et al, 2010), we do not know if the release of GM-CSF by the young tissue is a pre-requisite for the observed positive effect on plaque phagocytosis. To this end, we co-cultured young brain slices from GM-CSF knockout mice (GM-CSF-/-) with old APPPS1 brain slices. Under this experimental setup, we could not detect any decrease in amyloid plaque clearance. Correspondingly, we still observed an increase in CD68 coverage area when old tissue was co-cultured with GM-CSF-/- brain slices, suggesting that microglial numbers can be increased in the absence of GM-CSF secretion from the young tissue. These new data are presented in Figure 8D-F and discussed on the page 12 of the revised manuscript. Currently, we cannot exclude compensatory mechanisms in GM-CSF-/- mice that would lead to increased levels of other mitogenic factors (Bonfield et al, 2008; Shibata et al, 2001) and thereby to an underestimation of the relevance of this factor in our *ex vivo* model.

Comment 4: *CD68 may not necessarily be a marker of phagocytosis (page 5) but a more general marker of microglial reactivity*

Changes/Reply 4: We agree with the reviewer and modified accordingly our statement on page 5 of our manuscript (current page 6).

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Reviewer´s assessment (RA) Methods

Clodronate treatment of cultured astrocytes
Cortical astrocytes have been isolated from postnatal day 3 (P3) WT (C57Bl6J) mice as described previously (Kaech & Banker, 2006). Briefly, tissue has been dissociated first enzymatically (trypsin, Gibco) and later mechanically until obtaining homogenous cell suspension. Cells have been plated onto uncoated tissue culture plates and maintained in MEM (Gibco) supplemented with 0.6% Glucose and 5% FCS. Cells have been treated with increasing doses of clodronate (100 and 1000 µg/ml) added directly to the media, while PBS has been used as a vehicle control. Cell viability has been assessed 72h after treatment using lactate dehydrogenase (LDH) release assay (Promega) according to manufacturer´s instructions. Every treatment has been repeated at least in triplicates.

LDH measurements were performed in 2 independent experiments, each measurement was also done in triplicates. At the end of the treatment, cells have been PFA fixed and nuclei stained with DAPI as described in the manuscript. Representative pictures of all conditions have been acquired using a fluorescence microscope (Zeiss AxioImager A2) equipped with AxioCam MRm and AxioVision software package.

Biochemical analysis of Aβ40 and Aβ42 levels
RIPA lysates were prepared from 8 pooled slices and the remaining pellet after 14.000 g centrifugation (60 min at 4°C) was homogenized in 70% formic acid. The formic acid fraction was neutralized with 20 x 1 M Tris-HCl buffer (pH 9.5) and 10 µl were analyzed using self-made Wiltfang gels which allowed separation of Aβ40/Aβ42 species. Samples analyzed on Wiltfang gels were from the same extract as in Figure 2D of the manuscript. A mixture of synthetic Aβ38/Aβ40/Aβ42 peptides (AnaSpec; obtained from Eurogentec) was used as a control. Aβ detection was performed as described in the manuscript.

Sholl analysis
Images were converted to 8-bit color, isolated, and measured using the Fiji Sholl Analysis plug-in (http://fiji.sc/Sholl_Analysis). Specifically, the estimated geometric center was marked, the first circle was defined as a 10 µm radius for soma exclusion, and each outward concentric circle was an addition of 3 µm in radius. The linear profile of the plug-in was used to count the number of intersections and to calculate the Schoenen Ramification Index (SRI). SRI was defined as the ratio of the maximum number of branches to the number of primary branches (Nm/Nρ).

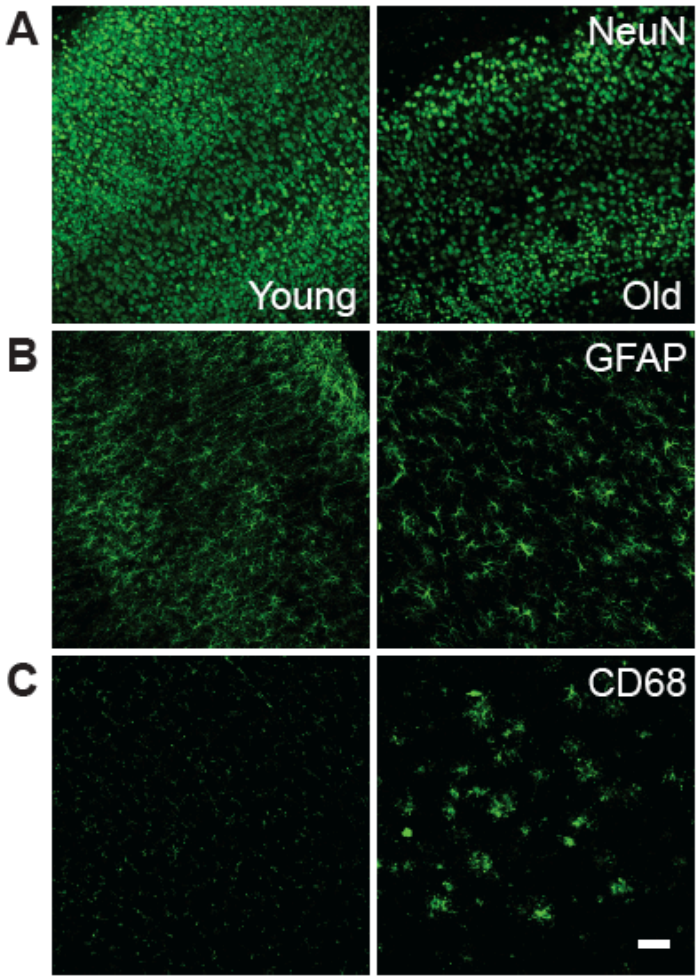

Figure RA1

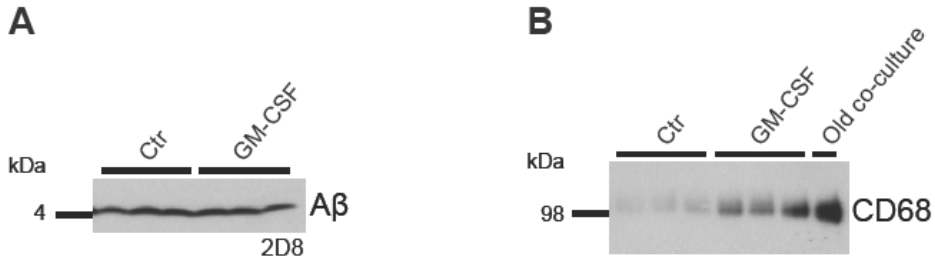

Figure RA2

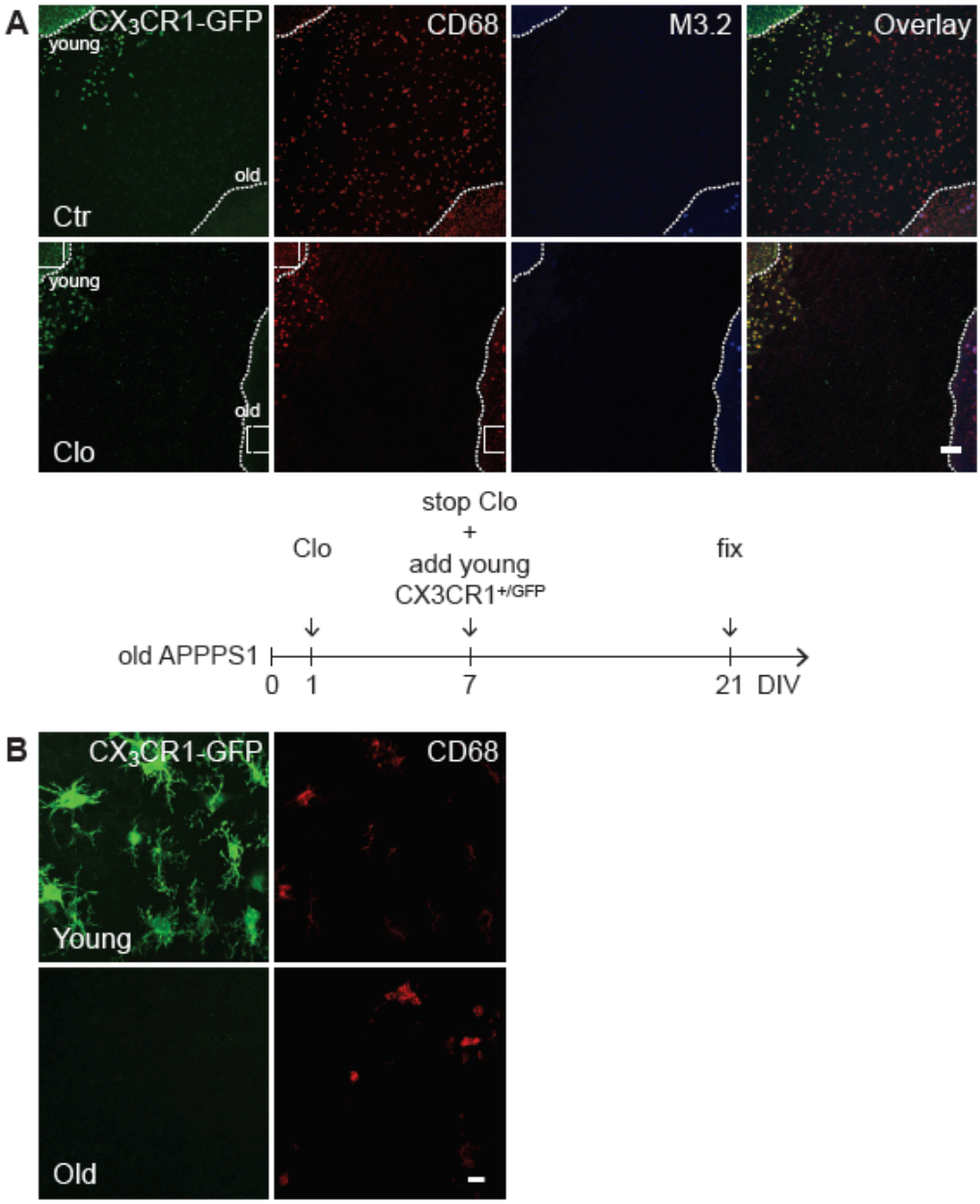

Figure RA3

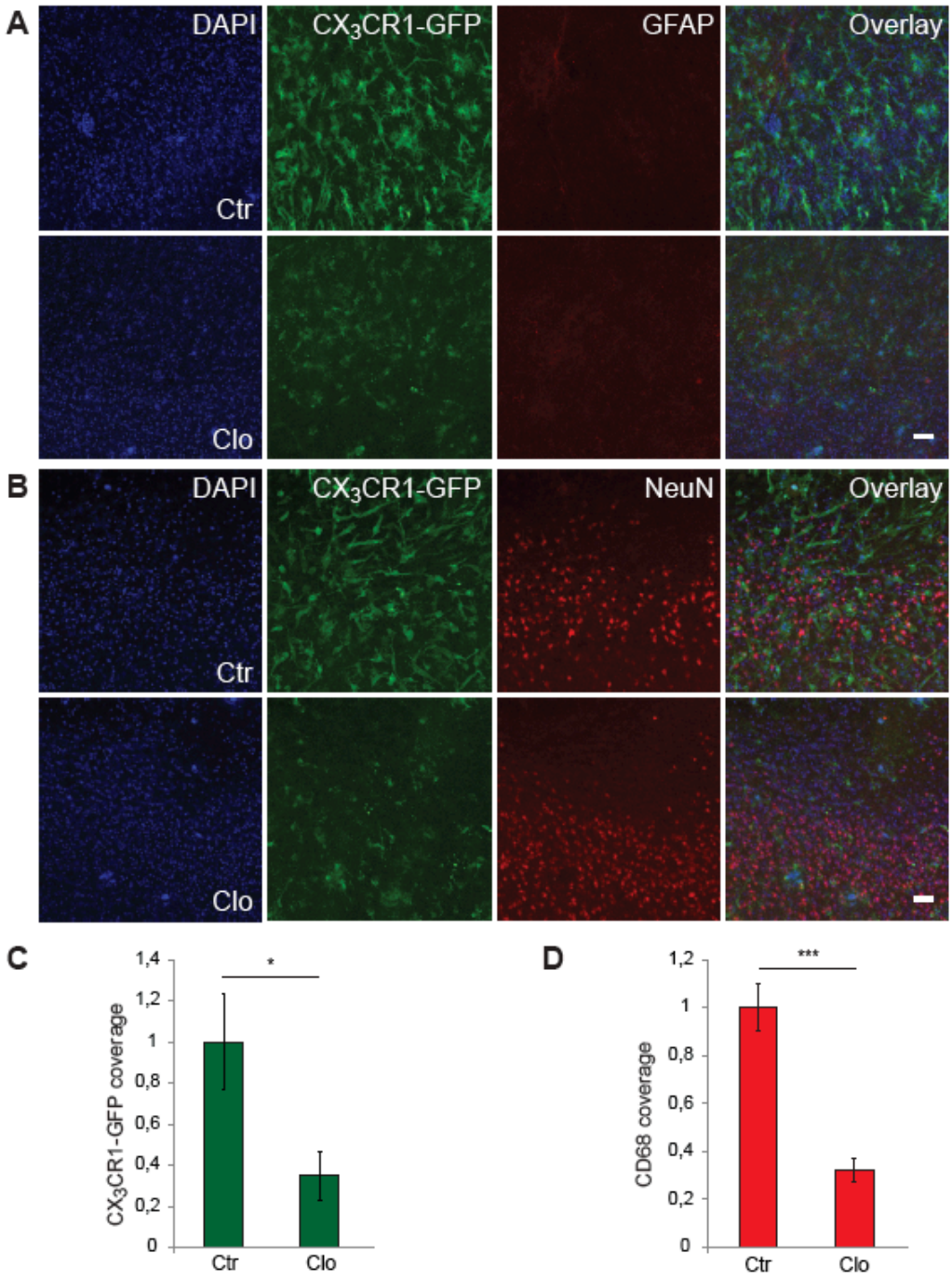
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Figure RA4

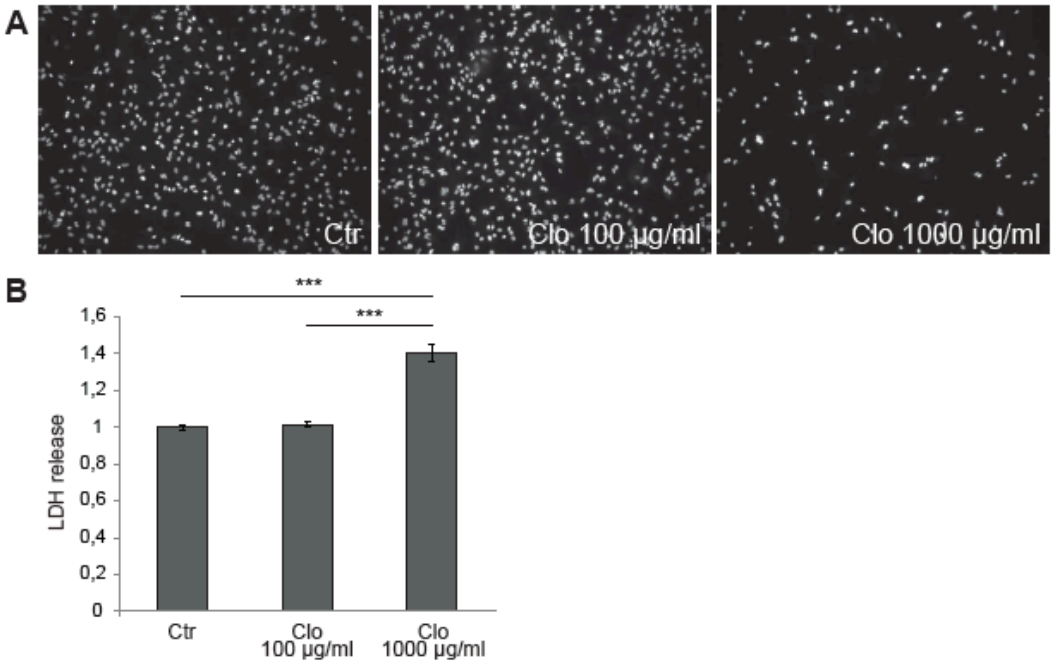

Figure RA5

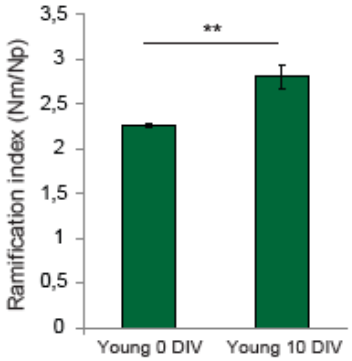

Figure RA6

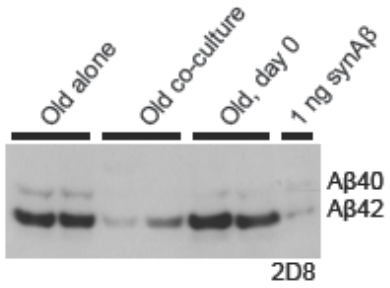

Figure RA7

Reviewer´s assessment (RA) Figure Legends

Figure RA1. Characterization of freshly cut brain slices.
(A-C) Immunofluorescence analysis of freshly cut (0 DIV) young WT and old APPPS1 brain slices using neuronal (NeuN), astrocytic (GFAP) and microglial (CD68) markers reveals gliosis that is a characteristic hallmark of aged APPPS1 brains and is manifested by activated GFAP and CD68 positive cells. Scale bar: 75 μm.

Figure RA2. Analysis of Aβ and CD68 levels in old APPPS1 tissue upon GM-CSF treatment.
Western blot analysis of Aβ **(A)** and CD68 **(B)** levels in old APPPS1 brain slices (triplicates) treated with GM-CSF and Ctr and cultured for 14 DIV.

Figure RA3. Young microglia are not infiltrating into the old APPPS1 slice upon clodronate treatment.
(A, B) Immunofluorescence analysis of co-cultured young CX3CR1+/GFP and Clo-treated old APPPS1 slices immunostained with GFP (green), CD68 (red) and 6E10 (blue). The old APPPS1 slice was treated with Clo and Ctr from 1 until 7 DIV and subsequently co-cultured with the young CX3CR1+/GFP slice for 14 DIV, as schematically indicated. We could detect some GFP-expressing young microglial cells that are migrating towards the old tissue, but could not detect any young microglial cells inside of the old tissue, similar to results presented in Figure EV4. Images of boxed regions in **A** are depicted at higher magnification in **B** and reveal that GFP positive microglial cells are indeed present in the young (upper panels) but not in the old (lower panels) brain slices. A GFP antibody was used to amplify the signal of CX3CR1-GFP positive young microglial cells. Scale bars: 100 μm **(A)** and 10 μm **(B)**.

Figure RA4. Clodronate treatment of old APPPS1 brain slices strongly reduces microglial numbers.
(A, B) Immunofluorescence analysis of the old APPPS1/CX3CR1+/GFP slice treated with Clo and Ctr from 1 until 7 DIV and immunostained with GFP (green) and the astrocytic marker GFAP (red) in **A** and with GFP (green) and the neuronal marker NeuN (red) in **B**. DAPI (blue) was used to counterstain nuclei. A GFP antibody was used to amplify the signal of GFP-expressing old microglial cells. Scale bar: 50 μm.
(C) Area of CX3CR1-GFP positive cells (CX3CR1-GFP coverage) in the old APPPS1/CX3CR1+/GFP tissue
treated with Clo and Ctr. CX3CR1-GFP coverage is strongly reduced upon Clo treatment. The values are normalized to CX3CR1-GFP coverage of the Ctr and represent mean ± SEM from 3 independent experiments, including total of 6 independent slice culture dishes (*$P < 0.05$).
(D) Area of CD68 positive cells (CD68 coverage) in the old APPPS1/CX3CR1+/GFP tissue treated with Clo and Ctr and subsequently immunostained with CD68. CD68 coverage is also strongly reduced upon Clo treatment, reflecting the CX3CR1-GFP coverage illustrated in **C**. The values are normalized to CD68 coverage of the Ctr and represent mean ± SEM from 3 independent experiments, including total of 6 independent slice culture dishes (***$P < 0.001$).

Figure RA5. Clodronate treatment of primary astrocytes.
Clodronate treatment of primary astrocytes at lower doses (100 μg/ml) does not cause toxicity in contrast to 10-fold higher doses (1000 μg/ml) that reduces cell viability. Cultured primary astrocytes were stained with DAPI to reveal nuclei (**A**) and analyzed using LDH release assay (**B**). The values are normalized to LDH release of the Ctr and represent mean ± SEM from 2 independent experiments. LDH measurements were done in triplicates (***$P < 0.001$).

Figure RA6. Sholl analysis of young microglia *ex vivo*.
Sholl analysis of young microglial cells in freshly cut CX3CR1+/GFP slices (0 DIV) and after culturing (10 DIV). The values indicate number of ramifications and represent mean ± SEM from 3 independent experiments, each experiment including 20 microglial cells (**$P < 0.01$).

Figure RA7. Aβ40 and Aβ42 levels are reduced upon co-culturing of old and young brain slices.
Western blot analysis of Aβ40 and Aβ42 levels (duplicates) in the old APPPS1 tissue cultured alone,

co-cultured with the young WT slices (14 DIV) or freshly cut (0 DIV) using 2D8 antibody. A mixture of synthetic Aβ peptides (1 ng) was used as a control for the successful separation of Aβ40 and Aβ42.

2nd Editorial Decision 18 November 2016

Thanks for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by the three referees and their comments are provided below.

As you can see from the comments, referees #1 and 3 appreciate the introduced revisions. Referee #2 is still not persuaded that the advance provided is sufficient to consider publication here. However, given the support provided by the two other referees I am pleased to say that we will accept the manuscript for publication here.

Before sending you the formal acceptance letter - I would like to give you the opportunity to provide a point-by-point response to the remaining comments of referee #2 if you wish. Also as this will be part of the review process file I think it would be good for you to respond. No changes are needed in the manuscript text. I have provided a revision link below for you to upload the response - this is the easiest way to do this. You can bring forth all the manuscript files.

REFEREE REPORTS

Referee #1:

The authors have addressed all my comments and suggestions appropriately. I think this now is an interesting paper suggesting that even old microglia can be induced to remove abeta by GMCSF. Good job.

Referee #2:

In this revised version of the paper there are now some details of the new ex vivo culture model. I find it hard to understand what we learn from this model that is relevant to AD. The authors now show, in contrast to the first version, that brain slices from 20 month old mice undergo massive neurodegeneration. By 7 days, the first analysis time point there is not only massive loss of neurons but also loss of astrocytes: the tissue slice must be little more than a soup of degenerating cells at 7 days, which progresses for a further 7 days as part of the analysis. The fact that there are macrophages with appearance of phagocytes, and the amyloid around the plaques is lost around the plaque cores is not surprising and it is hard to understand what is learnt of mechanistic relevance. The complete absence of the neuronal/glial microenvironment means that the macrophages that remain are very unlikely to have retained a phenotype that mimics the microglia of the aged or AD brain. The claim that "Continuous loss of neurons in our ex vivo model may trigger microglial activation similar to severe neurodegeneration in human AD brains" is far-fetched. I do not believe that this paper will provide any insight into microglia processing of amyloid in AD.

Referee #3:

I'm happy with the revised version and have no additional concerns that would preclude publication.

2nd Revision - authors' response 24 November 2016

Reply to comments from referee #2 on manuscript EMBOJ-2016-94591R:
“Young microglia restore amyloid plaque clearance of aged microglia”

Comments: *In this revised version of the paper there are now some details of the new ex vivo culture model. I find it hard to understand what we learn from this model that is relevant to AD. The authors now show, in contrast to the first version, that brain slices from 20 month old mice undergo massive neurodegeneration. By 7 days, the first analysis time point there is not only massive loss of neurons but also loss of astrocytes: the tissue slice must be little more than a soup of degenerating cells at 7 days, which progresses for a further 7 days as part of the analysis. The fact that there are macrophages with appearance of phagocytes, and the amyloid around the plaques is lost around the plaque cores is not surprising and it is hard to understand what is learnt of mechanistic relevance. The complete absence of the neuronal/glial microenvironment means that the macrophages that remain are very unlikely to have retained a phenotype that mimics the microglia of the aged or AD brain. The claim that "Continuous loss of neurons in our ex vivo model may trigger microglial activation similar to severe neurodegeneration in human AD brains" is far-fetched. I do not believe that this paper will provide any insight into microglia processing of amyloid in AD.*

Reply: As requested by the referee, we provided an in depth characterization of the newly established *ex vivo* model in the revised version of our manuscript. We agree with the referee that there is a massive loss of neurons and astrocytes in our *ex vivo* model. However, amyloid plaque clearance in our system can be enhanced (upon GM-CSF treatment and co-culturing) as well as inhibited (upon CytoD or AraC treatment) suggesting modulation of phagocytic capacity upon different experimental conditons which all experience same extent of cell loss. Thus, efficient clearance of amyloid plaques is not observed when old brain slices were maintained alone (14 DIV) in contrast to co-culturing of young and old brain slices and addition of young supernatant or GM-CSF to old brain slices. Furthermore, our study reveals that microglia indeed possess a high potential for repair of their phagocytic function - even in the presence of unfavorable cellular environment. It is possible that, as anticipated by the referee, cellular microenvironment of aged microglia *ex vivo* is not identical with AD brains. We have therefore omitted the statement *"Continuous loss of neurons in our ex vivo model may trigger microglial activation similar to severe neurodegeneration in human AD brains"* from the manuscript. However, the same criticism also applies to current models used to study microglial reaction to amyloid plaques in the complete absence of cell death. We strongly believe that neurodegeneration occuring in AD brains will influence microglial activity.

In addition, we would like to point out that by using this newly established *ex vivo* model it was possible for us to identify GM-CSF as a phagocytosis enhancing molecule which is fully in line with the published data and currently ongoing clinical trials for assessing GM-CSF as a possible amyloid reducing therapy via enhanced microglial phagocytosis (and proliferation). Those are exactly the same features that we observed upon GM-CSF addition to old brain slices and can be taken as a proof of principle for the suitability of this model for identifying and testing phagocytosis modifing compounds and studying potential for repair of microglial phagocytic capacity that is lost in AD.

EMBO PRESS

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Sabina Tahirovic, Christian Haass
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2016-94591

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen according to our experience with organotypic slice cultures and cell biological/biochemical experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No animal studies were conducted.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	No animal studies were conducted.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Analysis was done blinded to the experimental conditions.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No animal studies were conducted.
5. For every figure, are statistical tests justified as appropriate?	Yes. Statistical tests are mentioned in figure legends and further described in the Methods section (page 19, Statistical analysis).
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used the upaired two-tailed Student´s t-test as described on page 19.
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jij.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies were purchased by commercial sources and all catalog numbers are provided in the Methods section (page 15, Biochemical characterization of slice cultures and page 17, Immunohistochemistry). Exception is the Abeta antibody 2D8 (see citation in the Methods section, page 15, Biochemical characterization of slice cultures).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	.....tested for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Both male and female mice were used in our study. Detailed information is provided in the Methods section (page 14, section Animals).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	No animal studies were conducted.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	No animal studies were conducted.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	No experiments with human subjects were conducted.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	No experiments with human subjects were conducted.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	No experiments with human subjects were conducted.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	No experiments with human subjects were conducted.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	No experiments with human subjects were conducted.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	No experiments with human subjects were conducted.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	No experiments with human subjects were conducted.

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	No computational models were used.

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA