nature portfolio

Peer Review File

Clearance and Transport of Amyloid β by Peripheral Monocytes Correlate with Alzheimer's Disease Progression

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This study describes the role of circulating monocytes in the clearance of beta amyloid at the level of the BBB and the decrease phagocytic properties of these cells is associated with an increase cognitive decline in AD patients. They found a specific population of these cells, the intermediate monocytes that also seem to exhibit some characteristic of macrophages. Safe for the data on the brain, those of circulating monocytes are quite solid and very interesting. However, the identification of infiltrating cells in the brain is questionable and their numbers is highly variable. (The analysis indicated an average of 713 cells/mL in the 11 CSF samples, comprising 57.64±23.45% CD45+ peripheral immune cells and 40.43±23.55% CD45- cells and debris (Fig. 5k). Among CD45+ cells, an average of 309.8±74.8/mL lymphocytes and 79.0±30.6/mL monocytes were detected (Fig. 5a, typical case). ...). There is also no clear evidence that they are of systemic origin.

The second concern is the investigation of centrally injected cells that are therefore found in the circulation. First the number of cells is extremely low and second, they have been injected via the lateral ventricle. Such injection causes a major damage of the brain, which is associated with BBB breakdown. This may well have allowed the presence of few of these cells into the circulation without being a physiological event.

I would recommend focusing on the role of circulating monocytes in the phagocytosis of Abeta at the level of the luminal side of the BBB and the transport of Abeta across endothelial cells via LRP1, …

Specific comments

The authors mentioned that most of the Abeta is found at the surface of the cells and is not phagocyted. The assay to perform this is to expose cells to Abeta for 15 minutes. I think a longer period of exposure is absolutely needed to investigate this point.

The original study that investigated the role of circulating monocytes in the clearance of Abeta has not been cited.

Real-time in vivo imaging reveals the ability of monocytes to clear vascular amyloid beta. Michaud JP, Bellavance MA, Préfontaine P, Rivest S.Cell Rep. 2013 Nov 14;5(3):646-53. doi: 10.1016/j.celrep.2013.10.010. Epub 2013 Nov 7.PMID: 24210819 Free article.

Reviewer #2 (Remarks to the Author):

This is an interesting study, showing a reduction in abeta positive monocytes in MCI and AD patients, and binding of abeta to peripheral monocytes. It could become an impactful study, but first needs some further elaboration.

A major problem is that replication in an independent cohort is lacking, and that the power of the CSF analyses, relevant for the main conclusion, is very low. Moreover, the statistics applied are not proper, since the data show that they are not normally distributed. Lastly, it is difficult to follow. It now appears as a set of pilot experiments, and the interconnection between these experiments, and their rationale is not well explained. Moreover, there would benefit from more focussed and consistent experimentation to sustain the main conclusions. More work will help to convey the message better, which is potentially very interesting and can be groundbreaking.

Here are my detailed questions:

Can they exclude that the abeta in the peripheral monocytes is not taken up peripherally? This is not well addressed in the experiments nor in the discussion. What percentage of PBMCs is drained from the mice brain to the peripheral blood and what percentage to other tissues, or maybe a part is retained in the CSF?

Line 80-82: ''However, the precise mechanism through which peripheral monocytes participate in Aβ transportation across the blood-brain barrier (BBB)remains unclear.'' This sentence appears the knowledge gap to be addressed. I did not capture that from the study design presented in the abstract neither is it addressed in the results.

Relations between CSF and blood imply transport across the blood CSF barrier, but not across the BBB, which is the subject of the study.

Line 127, CSF monocytes were characterized with different markers than peripheral monocytes, namely CD45+, on top of cd14 and cd16. However, this is not introduced or explained.

Line 137: ''Some cells also expressed CD68, a marker for macrophage/activated microglia (Fig. 2e).'' this result was already revealed in the previous paragraph.

Did these monocytes uniquely express these receptors: other monocytes not (low abeta expressing ones)? Or at a lower level?

Line 151 and further: why was CCR2 used here as surface marker, and not abeta? there is likely a good rationale, but an introduction to that, and the research question, is needed.

172 and further: synthetic abeta antibody binding to PBMCs is shown. What is the question behind these experiments?

Is extracellular binding relevant? Is that a conceivable logical way of transportation of abeta out of the brain? Would that not rather be intra cellular phagocytosis? If intracellular abeta could be shown, that would be more convincing. The conclusions pertaining to the transport mechanism are therefore not sustained by the data.

Line 211, infiltrating monocytes are mentioned. What is the definition of infiltrating? Usually this term is used for monocytes infiltrating the parenchyma.
The mere presence of lymphocytes in the CSF is not novel and well established.

Line 227 -228: Thus, akin to circulating Aβ++ monocytes, these CSF monocytes are functionally akin to macrophages, demonstrating chemotaxis, migration, Aβ binding and phagocytosis.: actually, these functional capacities as chemotaix and migration are not shown by the experiments, only the presence of proteins that indicate the potential for such function.

The English writing needs to be improved throughout the manuscript, it is not easy to follow now. As already indicated, the results section lacks introduction into the goal of almost each experiment, and the headings should be more specific (correlation with abeta brain load: correlation of what with abeta brain load?). In addition, there are sometimes inaccurate sentences. An example of the latter: line 232 ''exhibited significant higher expression levels'' Higher expression levels of what exactly?
Furthermore, ''We conducted a statistical analysis'' line 258, is not a very informative stretch. One can assume that all comparisons so far have been done by statistical analyses.

Correlation with abeta load and cognitive scores: did the authors correlate the percentage of abeta-high monocytes in CSF or rather blood with these biological and cognitive scores?

For the enhanced discrimination of AD (again a more specific heading would be helpful), what

cohort was included and how complete was the dataset for this outcome? It is important to mention this in the text, to understand the power of the analyses.

Line 296, Upon integrating Aβ-associated biomarkers: which markers?

The mice experiments are interesting, but cannot provide definite proof of drainage via lymph nodes or other homeostatic pathways. This is because trauma induced by the injection could affect the behaviour of the monocytes. The experiments lack proper controls and detailed analysis of expression of relevant markers in these monocytes, and is a very exploratory.

Line 350: ''Remarkably, there have been no reports of macrophages existing in the circulation.'' This is not remarkably, but text book knowledge. Mature differentiated macrophages are only present within tissues.

Discussion lines 376 lacks references to support the more general statements.

Figure 6q, in the figure it should be written which 4 biomarkers are included.
The data look clearly not normally distributed in figure 6. Therefore, all statistics should be performed by other statistics than done now. This can potentially change the conclusions dramatically.

Reviewer #3 (Remarks to the Author):

We read the article by Huang and colleagues with great interest. They investigate the role of monocytes in clearance of AB from the brain via correlative models using PBMCs and CSF cell pellets across the continuum of AD from controls without significant accumulation of amyloid in the brain to dementia with amyloid. They use several antibodies to APP and Abetas and surface markers for immune cells. A major finding is the inverse association of intermediate monocytes carrying AB with amyloid load in the brain and cognitive function, suggesting that phagocytosis and clearance of amyloid from the brain may be impaired in AD. There is a small experiment in mice demonstrating entry of monocytes into peripheral blood of mice from brain compartment, however this no additional data suggesting that intermediate monocytes play a role in amyloid clearance from the brain of AD mouse models is not presented. This question is not entirely novel, but this is a valuable contribution to the literature in that cells are investigated in periphery and CSF in well characterized patients clinically and biologically (AD biomarkers). Overall, the authors investigate an important question especially in the era of amyloid-clearing therapeutics. The study is well designed and the manuscript well written. However, some aspects could be clarified and there's a concern regarding low numbers of samples in each group and imbalance in sex.

Major comments
- The authors are using AD in lieu of dementia which is incorrect. It should be CU, MCI, Dementia with and without positive AD biomarker status. In addition, the first time MCI is spelled it is incorrectly spelled out as "minor" rather than mild cognitive impairment.
- Major issue is small numbers and lack of validation cohort.
- Paragraph "Differential Aβ Peptide Interaction and Phagocytic Functions Across Peripheral Blood Monocyte Subsets": The authors correctly test antibodies against different epitopes of the APP peptide. However, all tested antibodies are non-specific for APP products, targeting early epitopes. Since, as the authors emphasize in the introduction, Aβ42 is the main amyloid product implicated in AD, it would be important to also test a Aβ42-specific antibody, since monocytes may target Aβ42 specifcially. It is intriguing that many of the cognitively normal participants also bear low levels of surface Aβ immunofluorescence and Aβ++ intermediate monocytes, overlapping with the MCI and AD groups (Figure 6). This is not addressed in the discussion and not addressed in the animal experiment. We recommend that they show exactly what part of APP and its peptides is detected by each antibody graphically.
- The number of samples from which CSF cell pellets were collected is very small (n=11) to draw clear conclusions. The p chart Fig 5.k is confusing what does Total =1 mean?
- Paragraph "Aβ++ Cells in CSF": There is the possibility that CSF pellets also included microglial

cells, which could confound later results. Despite CD45 expression being relatively low in resting state microglia, it has been suggested that some conditions, including AD, might induce increase of CD45 expression in brain resident myeloid cells (Refs 1, 2, 5). The other markers that were used for characterization (CD14, CD16, CX3CR1, CCR2) have also been shown to be expressed by microglia. Therefore their distinction based on the used markers may be difficult. The authors should try to identify which proportion of the isolated CSF cells consists of microglia by using more highly specific microglial markers. Another approach could be to further characterize CSF PBMCs with CD163 that has been suggested to be specific for peripheral infiltrating monocytes (Ref 5). Additionally, an experiment in a mouse model of amyloidosis with labelled peripheral monocytes and their isolation from CSF would provide strong translational evidence for the validation of this finding.
- Previous studies that have investigated Aβ – monocyte interactions are not being discussed and should be compared to the results in this work (Ref 3). Importantly, this work also examined subpopulations of monocytes based on CD14 and CD16 status. Furthermore, the authors should discuss pathology studies that support the role of peripheral monocytes in amyloid clearance (Ref 4, 5). These studies also provide evidence for increased expression of some of the markers that were identified to be increased in Aβ-associated monocytes (CCR2, CD68).
- It is hard to follow all the percentages and numbers. We suggest summarizing major findings graphically and with clearer data visualization
- In mouse experiment it is surprising that young mice had a lower detection rate than older mice—seemingly opposite to what would be anticipated based on human data
- Demographic table has control and case columns and in control there is AD (which should be dementia) and MCI. The use of AD biomarker negative cognitively impaired controls is helpful and should be addressed in the text and also findings in this group clearly discussed.
- a deeper characterization of cells with scRNAseq would be a great addition
- A graphical abstract is needed

Minor comments
- YO beads should be spelled out
- Line 62: we suggest changing “AD’s underlying pathogenic mechanisms” to “AD’s possible underlying pathogenic mechanisms” since the modest success of the latest anti-amyloid treatments have created disputes about the amyloid hypothesis.
- Minor typographical error in line 63: “originated” ◊ “originates”.
- Some sentences and paragraphs should be re-written for clarity e.g.: line 230—which group? Line 232 what is meant by “expression level”? paragraph starting line 263 what’s the difference with prior paragraph?
- Line 283, why not provide AUC, similar to what follows?
- Line 294—not clear what model is being considered?

References
1. Honarpisheh P et al. Potential caveats of putative microglia-specific markers for assessment of age-related cerebrovascular neuroinflammation. J Neuroinflammation. 2020 Dec 1;17(1):366. doi: 10.1186/s12974-020-02019-5.
2. Masliah E et al. Immunoreactivity of CD45, a protein phosphotyrosine phosphatase, in Alzheimer's disease. Acta Neuropathol. 1991;83(1):12-20. doi: 10.1007/BF00294425.
3. Chen SH et al. Amyloid-beta uptake by blood monocytes is reduced with ageing and Alzheimer's disease. Transl Psychiatry. 2020 Dec 8;10(1):423. doi: 10.1038/s41398-020-01113-9.
4. Yan P et al. Peripheral monocyte-derived cells counter amyloid plaque pathogenesis in a mouse model of Alzheimer's disease. J Clin Invest. 2022 Jun 1;132(11):e152565. doi: 10.1172/JCI152565.
5. Muñoz-Castro C et al. Monocyte-derived cells invade brain parenchyma and amyloid plaques in human Alzheimer's disease hippocampus. Acta Neuropathol Commun. 2023 Feb 28;11(1):31. doi: 10.1186/s40478-023-01530-z. PMID: 36855152; PMCID: PMC9976401.

Reviewer #4 (Remarks to the Author):

In this manuscript, Huang and co-workers examine the correlation between Aβ++ monocytes in blood and CSF, brain Aβ burden by PET and cognitive decline in MCI and AD. They report a negative correlation between monocyte carriage of Aβ and cognitive decline/brain amyloid β accumulation in AD. They also propose that fluorophore-tagged PBMCs can be detected in deep cervical lymph nodes and blood after intraventricular injection in APP/PS1 mice. The topic is interesting but the results presented in this manuscript are often difficult to interpret and no conclusive evidence for clearance of Aβ by monocytes is provided.

Major points:

• The authors need to demonstrate which epitope(s) the antibody W0-2 binds to on monocytes and demonstrate specificity.
• The authors should characterize the small fraction of Aβ++ intermediate monocytes more carefully on a transcriptomic level.
• The correlations between Aβ++ monocytes in blood and CSF, brain Aβ burden by PET and cognitive decline in MCI and AD in Fig. 6 are quite weak.
• The sensitivity of the base model is low and statistical significance is not reached (Fig. 6).
• Abstract: Strange to call the cells "Aβ expressing monocytes" (line 34) if the point is that they have bound to it/consumed it for clearance purposes, rather than producing it themselves.
• The number of independent biological replicates is extremely unclear throughout. This information should be provided in figure legends and made clearer in figures. Few convincing quantitative analyses have been performed.
o Figure 1: Very little information given about N numbers. Good to show representative plots (if these are representative) but why has no quantification been performed on anything? Where is quantification to support the claim that "the majority of Aβ resided on the surface of CD14+CD16+ monocytes"? The surface Aβ staining doesn't look like surface staining.
o Figure 2: It is not clear what stats were performed and how significance was determined. No information in figure legend. The plots you are finding significance for (e.g. D, F, G) have values running off the graph (as discussed further below) so how can you accurately report on their fluorescent values to perform comparisons?
o N number for supplementary figure 3 unclear. How many replicates is this based on? Why are the highest expressing CD14+ cells not included in the gates in b? Same issue about lack of clarity for N numbers throughout supplementary figures 1-5. Statistics in supplementary figure 3?
o In cases where data values for individual replicates are available (e.g. in supplementary table 1 related to figure 7), plots from the most extreme cases appear to have been used, rather than representative plots (as discussed further below).
• In many cases, flow scale problems suggest incorrect voltage settings were used. All fluorophore signals should fall within the plot.
o For examples, Supplementary Fig 1: Why are the cells with the highest signal off the scale (in the green boxes)? It makes it difficult to interpret.
o Figure 1a, 1c and Figure 2: Again, flow scale problems suggesting incorrect voltage settings used. All fluorophore signals should be within the plot.
o Figure 4: Data running off plots again so very difficult to interpret the stats.
• Figure 7: Why are no statistical comparisons or quantitative results provided from the different mice? Based on the supplementary table provided, the authors have decided to show the plots from the mouse with the highest number of "migrating" cells in the figure (i.e. 42 cells out of 666,000 in the lymph node), rather than a more representative plot. In general, the numbers of cells detected are extremely low – one of the samples the authors are using to claim for peripheral migration of the injected cells to lymph nodes includes a mouse with only 1 positive cell out of 817,000 events – and the results are similar for the other samples. The same is true for the blood data (e.g. 1 cell out of 1 million events for sample 9). This is not convincing and one could just as easily imagine someone making the exact opposite conclusion (i.e. lack of migration) based on these data.
o Related to this, the authors claim in the discussion that "Over a two-day period, an average of 11 cells migrated into peripheral blood and dcLNs, indicating that monocytes have the capacity to exit the CNS and re-enter the periphery (Fig. 7)". Where is this average of 11 coming from? Based

on the supplementary table provided, the average for blood is 3.8 cells and the average for dcLNs is 7.7 cells. This is another example of extremely puzzling reporting.
• Several points in the discussion are puzzling.
o For example, what do you mean when you say "Our research, in line with others, discovered the presence of peripheral leukocytes in the CNS"?
o The following claims are too strong, as the functional importance of these Aβ- carrying cells is not shown and is based on correlations "Our study underscores the vital role of peripheral monocytes in the pathogenesis of AD, serving as essential players in the phagocytosis and transportation of Aβ peptides between the CNS and periphery"
• In lines 127-129: The authors state "Similarly, in CSF, the majority of CD45+CD14+CD16+ monocytes also express CD68 and Trem2 (Fig. 1c-d)". You have analysed extremely few cells from the CSF (Figure 1d). And why do you call these monocytes if they have macrophage phenotypes? CD14 and CD16 are also expressed by certain CNS macrophages, such as some reactive and plaque-associated human microglia (see, for example, PMID: 8227309 and PMID: 26286145).

Minor points:

o No legend is provided for supplementary figure 5.

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

RE: REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This study describes the role of circulating monocytes in the clearance of beta amyloid at the level of the BBB and the decrease phagocytic properties of these cells is associated with an increase cognitive decline in AD patients. They found a specific population of these cells, the intermediate monocytes that also seem to exhibit some characteristic of macrophages. Safe for the data on the brain, those of circulating monocytes are quite solid and very interesting. However, the identification of infiltrating cells in the brain is questionable and their numbers is highly variable. (The analysis indicated an average of 713 cells/mL in the 11 CSF samples, comprising 57.64±23.45% CD45+ peripheral immune cells and 40.43±23.55% CD45- cells and debris (Fig. 5k). Among CD45+ cells, an average of 309.8±74.8/mL lymphocytes and 79.0±30.6/mL monocytes were detected (Fig. 5a, typical case). ...). There is also no clear evidence that they are of systemic origin.

RE: We appreciate the Reviewer's keen interests and positive comments in our work. The data on infiltrating leukocytes in CSF aims to demonstrate several key points: 1) the presence of circulating leukocytes in CSF, characterized by $CD45^+$ (the pan-peripheral leukocyte surface biomarker); 2) this infiltration occurs in all participants studies, whether CU or with AD, despite significant variation in cell counts due to individual differences; 3) the high frequency of $CD45^+CD14^+CD16^+$ monocytes in CSF, compared to their low percentage in circulation, suggests that these cells are more likely to infiltrate the CNS. The data from 11 fresh CSF samples support these findings, indicating that such infiltration could be a regular pathway for monocytes participating in Aβ clearance in the brain. Although we made efforts to obtain more CSF samples, we were only able to include four additional samples in this revision due to the known difficulties associated with sample collection.

The second concern is the investigation of centrally injected cells that are therefore found in the circulation. First the number of cells is extremely low and second, they have been injected via the lateral ventricle. Such injection causes a major damage of the brain, which is associated with BBB breakdown. This may well have allowed the presence of few of these cells into the circulation without being a physiological event.

I would recommend focusing on the role of circulating monocytes in the phagocytosis of Abeta at the level of the luminal side of the BBB and the transport of Abeta across endothelial cells via LRP1, …

RE: We acknowledge the possibility that cells injected into the lateral ventricle may leak into circulation via a damaged blood-brain barrier (BBB). However, our data show only small numbers of injected cells in circulation (1-21 in 0.3-1.5×10^6 leukocytes), and 5 out of 18 mice showed no cells two days after injection, making it unlikely that these cells migrated into circulation via BBB leakage. Additionally, we performed additional experiments by injecting fluorescent beads into the ventricle, and none of the beads were found in either circulation or lymph nodes (see updated Suppl. Table 1), indicating that the cell migration we observed is a physiological process.

We have also considered the hypothesis regarding the clearance of Aβ from the luminal side of the brain vasculature. Thank you for mentioning Michaud et al.'s work; we have previously read that paper and found it to be quite inspiring. However, it is important to note that this hypothesis does not fully account for the presence of monocytes that we identified in the CSF. To address this complex phenomenon, we used challenging tracing techniques to propose an alternative possibility.

As indicated in the manuscript, these cells may traverse the blood-brain barrier or could potentially return to the periphery via brain lymphatic drainage, a concept recently discussed in the work of Yoon et al. (2024). If this hypothesis proves to be accurate, it would provide an explanation for the entry and exit mechanisms of these immune cells between the periphery and the CSF. This directly links the peripheral and central immune systems, constituting a significant and noteworthy discovery.

Reference:

Yoon, JH., Jin, H., Kim, H.J. et al. Nasopharyngeal lymphatic plexus is a hub for cerebrospinal fluid drainage. Nature (2024). https://doi.org/10.1038/s41586-023-06899-4

Specific comments

The authors mentioned that most of the Abeta is found at the surface of the cells and is not phagocyted. The assay to perform this is to expose cells to Abeta for 15 minutes. we think a longer period of exposure is absolutely needed to investigate this point.

RE: Inspired by the Reviewer's suggestion, we have recently developed a Two-Color Fluorescent Reporting System for the real-time monitoring of microglial phagocytosis of oligomerized Aβ (oAβ). This system incorporates AF647-conjugated Aducanumab to visualize surface-bound Aβo, while pHrodo red is used to highlight intra-lysosomal Aβo. To extend the exposure period, we employed flow cytometry at multiple time points (0.5, 1, 2, 4, and 24 hours) for continuous observation. These data have been included in the revision.

These results provide preliminary support for our hypothesis, and we are dedicated to gathering additional data to further validate and reinforce these findings in the forthcoming article.

The original study that investigated the role of circulating monocytes in the clearance of Abeta has not been cited.

Real-time in vivo imaging reveals the ability of monocytes to clear vascular amyloid beta. Michaud JP, Bellavance MA, Préfontaine P, Rivest S.Cell Rep. 2013 Nov 14;5(3):646-53. doi: 10.1016/j.celrep.2013.10.010. Epub 2013 Nov 7.PMID: 24210819 Free article.

RE: We have cited this work in the revision.

Reviewer #2 (Remarks to the Author):

This is an interesting study, showing a reduction in abeta positive monocytes in MCI and AD patients, and binding of abeta to peripheral monocytes. It could become an impactful study, but first needs some further elaboration.

A major problem is that replication in an independent cohort is lacking, and that the power of the CSF analyses, relevant for the main conclusion, is very low. Moreover, the statistics applied are not proper, since the data show that they are not normally distributed. Lastly, it is

difficult to follow. It now appears as a set of pilot experiments, and the interconnection between these experiments, and their rationale is not well explained. Moreover, there would benefit from more focussed and consistent experimentation to sustain the main conclusions. More work will help to convey the message better, which is potentially very interesting and can be groundbreaking.

RE: We greatly appreciate the Reviewer's understanding of the significance and potential impact of this work and agree with the limitations pointed by the Reviewer. Great endeavour has been made to improve this work, which are shown in the revised version. More experimental work has been performed to better support our findings and the rationale/hypothesis. Additionally, we have improved our statistical methods by using nonparametric methods for data that are not normally distributed. Regarding the replication of an independent cohort, since this work mainly focuses on novel discoveries rather than clinical diagnostics, a validation cohort may not be necessary.

Here are my detailed questions:

Can they exclude that the abeta in the peripheral monocytes is not taken up peripherally? This is not well addressed in the experiments nor in the discussion. What percentage of PBMCs is drained from the mice brain to the peripheral blood and what percentage to other tissues, or maybe a part is retained in the CSF?

RE: Given current technological limitations, determining whether the Aβ in monocytes originates from the periphery or from the CNS remains challenging. However, existing clues suggest that Aβ on monocytes is more likely from the CSF: 1) Aβ concentration is about 15 times greater in CSF than in peripheral blood [7-10]; 2) we observed a significantly higher percentage of Aβ-adhering monocytes in CSF compared with in blood; 3) monocytes are professional phagocytes, which uptake Aβ, particularly Aβ oligomers/fibrils, via the innate phagocytic pathway. Previously we have shown that innate phagocytosis can be inhibited by as little as 1% of serum (Gu et al, *J Biol Chem* 287:17318). Therefore, it is unlikely that monocytes could efficiently clear Aβ in the peripheral. We have revised our Discussion on this topic.

Our experiments injecting cells into the ventricle showed that only a very small percentage of injected leukocytes were drained from the CNS to the lymph nodes or peripheral blood, less than 0.1%, with the majority retained in the CNS of the mice. This finding is consistent with our observations in human CSF and blood.

Line 80-82: ‘’However, the precise mechanism through which peripheral monocytes participate in Aβ transportation across the blood-brain barrier (BBB) remains unclear.’’ This sentence appears the knowledge gap to be addressed. we did not capture that from the study design presented in the abstract neither is it addressed in the results.

BG: We have modified the sentence to better address the focus of this study.

Relations between CSF and blood imply transport across the blood CSF barrier, but not across the BBB, which is the subject of the study.

RE: We agree with the Review’s opinion. The blood-CSF barrier at the choroid plexus, established by epithelial cells and their interconnected tight junctions, may play a significant role in monocyte migration between the blood and CSF. This barrier is unidirectional and influenced by blood flow and pressure to generate CSF. As outlined in the manuscript, the cells under consideration might traverse the blood-CSF barrier, the blood-brain barrier and potentially return to the periphery through brain lymphatic drainage, a concept recently explored in the research by Yoon et al. (2024).

Reference:

Yoon, JH., Jin, H., Kim, H.J. et al. Nasopharyngeal lymphatic plexus is a hub for cerebrospinal fluid drainage. Nature (2024). https://doi.org/10.1038/s41586-023-06899-4

Line 127, CSF monocytes were characterized with different markers than peripheral monocytes, namely CD45+, on top of cd14 and cd16. However, this is not introduced or explained.

RE: CD45 is a widely recognized marker for peripheral blood immune cells, which enhances the robustness of our immunophenotyping analyses. This addition contributes to the

credibility of characterizing immune cell populations in the CSF. We have provided further explanation about the importance of CD45 within this context.

Line 137: ‘’Some cells also expressed CD68, a marker for macrophage/activated microglia (Fig. 2e).’’ this result was already revealed in the previous paragraph.

RE: We have removed the duplicated result description.

Did these monocytes uniquely express these receptors: other monocytes not (low abeta expressing ones)? Or at a lower level?

RE: To the best of our knowledge, these $A\beta^{++}CD14^{+}CD16^{+}$ monocytes uniquely express CD68 and TREM2 (Fig. 4), while the other receptors shown in Figure 4 are expressed to varying degrees in different subsets of monocytes (Suppl. Fig. 4). Our recent work (Huang et al, *Alzheimer's & Dementia, 19:2084, 2022*) has reported the differential expression levels of some of these receptors in leukocytes from AD patients and CU controls.

Line 151 and further: why was CCR2 used here as surface marker, and not abeta? there is likely a good rationale, but an introduction to that, and the research question, is needed.

RE: CCR2 was chosen as a surrogate marker for cell sorting due to the use of the anti-Aβ antibody W0-2 in the following Western blotting and mass spectrometry analyses. We have included further clarification to elucidate the rationale behind opting for CCR2 in this context.

172 and further: synthetic Abeta antibody binding to PBMCs is shown. What is the question behind these experiments?

RE: The objectives of these experiments are: 1) to determine whether synthetic Aβ binds to different leukocyte types, such as monocytes, lymphocytes, or neutrophils, with different affinities (Fig. 2); 2) to investigate whether synthetic Aβ binds to different monocyte subsets with different affinities; 3) to establish that the binding profile observed in these experiments closely resembles the *in vivo* scenario of Aβ binding to monocytes.

Is extracellular binding relevant? Is that a conceivable logical way of transportation of Abeta out of the brain? Would that not rather be intra cellular phagocytosis? If intracellular Abeta could be shown, that would be more convincing. The conclusions pertaining to the transport mechanism are therefore not sustained by the data.

RE: Phagocytosis process includes capture, internalization and phagosome/lysosome fusion. Cell surface adhesion is the first evidence for clearance of oligomerized Aβ (oAβ). It shows that Aβ has been captured by scavenger receptors on the cell surface. The intracellular oAβ can be difficult to detect as it undergoes rapid proteolysis during phagosome/lysosome fusion. To address this concern, we have recently implemented a Two-Color Fluorescent Reporting System for real-time monitoring of microglial interactions with pHrodo labelled Aβ oligomers. In this system, AF647-conjugated Aducanumab is used to visualize surface-bound oAβ, while pHrodo red highlights intra-lysosomal oAβ. To extend the exposure period, we employed flow cytometry at multiple time points (0.5, 1, 2, 4, and 24 hours) for continuous observation. The new data has been incorporated into the revised version.

Line 211, infiltrating monocytes are mentioned. What is the definition of infiltrating? Usually this term is used for monocytes infiltrating the parenchyma.

RE: These monocytes found in the CSF express the pan peripheral leukocyte marker CD45, indicating that they originated from the blood, not the CNS. Therefore, we believe they are cells that have infiltrated into the CSF and potentially the parenchyma. The new data from brain samples has been incorporated into the revised version.

The mere presence of lymphocytes in the CSF is not novel and well established.

RE: Although the presence of lymphocytes in the CSF is widely recognized, our focus here is on monocytes, particularly the significantly higher proportion of $CD14^{+}CD16^{+}$ monocytes in the CSF compared to their occurrence in peripheral blood.

Line 227 -228: Thus, akin to circulating Aβ++ monocytes, these CSF monocytes are functionally akin to macrophages, demonstrating chemotaxis, migration, Aβ binding and

phagocytosis.: actually, these functional capacities as chemotaix and migration are not shown by the experiments, only the presence of proteins that indicate the potential for such function.

RE: The limited cell number in human CSF samples does present a challenge for conducting functional tests, including chemotaxis and migration. However, the high expression of multiple types of chemotaxis receptors suggests the potential for such functional capacities.

The English writing needs to be improved throughout the manuscript, it is not easy to follow now. As already indicated, the results section lacks introduction into the goal of almost each experiment, and the headings should be more specific (correlation with abeta brain load: correlation of what with abeta brain load?). In addition, there are sometimes inaccurate sentences. An example of the latter: line 232 ‘’exhibited significant higher expression levels’’ Higher expression levels of what exactly?

RE: We have gone through extensive revision on the entire manuscript to better describe our work. We have made necessary adjustments to improve the flow of the text and provide more specific introductions to the goals of each experiment. Headings have also been incorporated. Additionally, we have addressed inaccuracies in sentence construction, including the instance you highlighted.

Furthermore, ‘’We conducted a statistical analysis’’ line 258, is not a very informative stretch. One can assume that all comparisons so far have been done by statistical analyses.

RE: We have removed this redundant description accordingly.

Correlation with abeta load and cognitive scores: did the authors correlate the percentage of abeta-high monocytes in CSF or rather blood with these biological and cognitive scores?

RE: We have clarified this in the revision.

For the enhanced discrimination of AD (again a more specific heading would be helpful), what cohort was included and how complete was the dataset for this outcome? It is important to mention this in the text, to understand the power of the analyses.

RE: The cohort used for the enhanced discrimination of AD is the same as the one utilized in the previous analyses. To enhance clarity, we clarified this in the revision.

Line 296, Upon integrating Aβ-associated biomarkers: which markers?

RE: The specific Aβ-associated biomarkers were mentioned in the previous paragraph: "Subsequently, a panel comprising four biomarkers, including the percentage of $A\beta^{++}$ monocytes, the percentage of $A\beta^{+}$ NK T cells, the percentage of $A\beta^{++}$ classical monocytes, and the percentage of intermediate monocytes, outperformed other examined biomarker combinations."

The mice experiments are interesting, but cannot provide definite proof of drainage via lymph nodes or other homeostatic pathways. This is because trauma induced by the injection could affect the behaviour of the monocytes. The experiments lack proper controls and detailed analysis of expression of relevant markers in these monocytes, and is a very exploratory.

RE: We have conducted additional control experiments using fluorescent beads (6 µm high intensity alignment green beads, BD) injected into the lateral ventricle of APP/PS1 mice as a substitute for monocytes. Two days after injection, we did not detect any of these beads in peripheral blood or lymph nodes in three mice (aged 32 weeks, 48 weeks, and 55 weeks) . We have incorporated the results in Suppl. Table 1 and Suppl. Fig. 10.

Line 350: ''Remarkably, there have been no reports of macrophages existing in the circulation.'' This is not remarkably, but text book knowledge. Mature differentiated macrophages are only present within tissues.

RE: The presence of macrophage-like monocytes in the circulation is a novel finding in this study.

Discussion lines 376 lacks references to support the more general statements.

RE: We have incorporated more references.

Figure 6q, in the figure it should be written which 4 biomarkers are included.

The data look clearly not normally distributed in figure 6. Therefore, all statistics should be performed by other statistics than done now. This can potentially change the conclusions dramatically.

RE: We appreciate your clarification. Accordingly, we have consistently employed Spearman correlation analysis, a non-parametric method. In cases where data is not normally distributed, we have replaced ordinary ANOVA with Kruskal-Wallis test. This ensures that our statistical approach aligns appropriately with the characteristics of the data at hand.

Reviewer #3 (Remarks to the Author):

We read the article by Huang and colleagues with great interest. They investigate the role of monocytes in clearance of AB from the brain via correlative models using PBMCs and CSF cell pellets across the continuum of AD from controls without significant accumulation of amyloid in the brain to dementia with amyloid. They use several antibodies to APP and Abetas and surface markers for immune cells. A major finding is the inverse association of intermediate monocytes carrying AB with amyloid load in the brain and cognitive function, suggesting that phagocytosis and clearance of amyloid from the brain may be impaired in AD. There is a small experiment in mice demonstrating entry of monocytes into peripheral blood of mice from brain compartment, however this no additional data suggesting that intermediate monocytes play a role in amyloid clearance from the brain of AD mouse models is not presented. This question is not entirely novel, but this is a valuable contribution to the literature in that cells are investigated in periphery and CSF in well characterized patients clinically and biologically (AD biomarkers). Overall, the authors investigate an important question especially in the era of amyloid-clearing therapeutics. The study is well designed and the manuscript well written. However, some aspects could be clarified and there's a concern regarding low numbers of samples in each group and imbalance in sex.

Major comments

- The authors are using AD in lieu of dementia which is incorrect. It should be CU, MCI, Dementia with and without positive AD biomarker status. In addition, the first time MCI is spelled it is incorrectly spelled out as "minor" rather than mild cognitive impairment.

RE: We have revised them.

- Major issue is small numbers and lack of validation cohort.

RE: The major focus of this work is on the characterization of identified Aβ-binding monocyte in the circulation and their potential role in clearance of Aβ. We have changed the term “application” in clinical diagnosis to “implication”.

- Paragraph “Differential Aβ Peptide Interaction and Phagocytic Functions Across Peripheral Blood Monocyte Subsets”: The authors correctly test antibodies against different epitopes of the APP peptide. However, all tested antibodies are non-specific for APP products, targeting early epitopes. Since, as the authors emphasize in the introduction, Aβ42 is the main amyloid product implicated in AD, it would be important to also test a Aβ42-specific antibody, since monocytes may target Aβ42 specifcially. It is intriguing that many of the cognitively normal participants also bear low levels of surface Aβ immunofluorescence and Aβ++ intermediate monocytes, overlapping with the MCI and AD groups (Figure 6). This is not addressed in the discussion and not addressed in the animal experiment. We recommend that they show exactly what part of APP and its peptides is detected by each antibody graphically.

RE: We have included a sketch map illustrating the binding domains of the antibodies used. It's important to note that in the synthetic Aβ peptide binding test, both Aβ1-42 and Aβ1-40 were tested, and there is currently no evidence supporting the idea that monocytes may specifically target Aβ1-42. We have provided additional analysis on CU individuals with Aβ-PET above 25 CL in the revision.

- The number of samples from which CSF cell pellets were collected is very small (n=11) to draw clear conclusions. The p chart Fig 5.k is confusing what does Total =1 mean?

RE: We did our best to obtain more CSF pellet cell samples, due to the known difficulties, we are only able to add four more samples in this revision. In the pie chart, 1 corresponds to 100% of the total.

- Paragraph “Aβ++ Cells in CSF”: There is the possibility that CSF pellets also included microglial cells, which could confound later results. Despite CD45 expression being relatively low in resting state microglia, it has been suggested that some conditions, including AD, might induce increase of CD45 expression in brain resident myeloid cells (Refs 1, 2, 5). The other markers that were used for characterization (CD14, CD16, CX3CR1, CCR2) have also been shown to be expressed by microglia. Therefore their distinction based on the used markers may be difficult. The authors should try to identify which proportion of the isolated CSF cells consists of microglia by using more highly specific microglial markers. Another approach could be to further characterize CSF PBMCs with CD163 that has been suggested to be specific for peripheral infiltrating monocytes (Ref 5). Additionally, an experiment in a mouse model of amyloidosis with labelled peripheral monocytes and their isolation from CSF would provide strong translational evidence for the validation of this finding.

RE: We have contacted BioLegend to customize a fluorescent dye-conjugated TMEM119, a known microglia-specific marker, which holds promise for improving the accuracy of microglial cell identification. Additionally, we have included CD163 for further characterization of CSF monocytes.

We tried collecting mouse CSF using a specialized mouse CSF collection plate connected to an isoflurane machine. This method allows for collecting approximately 10 µL of CSF from one animal. Therefore, it is technically difficult to investigate mouse CSF monocytes.

- Previous studies that have investigated Aβ – monocyte interactions are not being discussed and should be compared to the results in this work (Ref 3). Importantly, this work also examined subpopulations of monocytes based on CD14 and CD16 status. Furthermore, the authors should discuss pathology studies that support the role of peripheral monocytes in amyloid clearance (Ref 4, 5). These studies also provide evidence for increased expression of some of the markers that were identified to be increased in Aβ-associated monocytes (CCR2, CD68).

RE: We find it remarkable that different studies converge on similar findings. We have incorporated those reference in our discussion.

- It is hard to follow all the percentages and numbers. We suggest summarizing major findings graphically and with clearer data visualization

RE: We appreciate your suggestion for clearer data visualization.

- In mouse experiment it is surprising that young mice had a lower detection rate than older mice—seemingly opposite to what would be anticipated based on human data

RE: The differences in detection rate may be attributed to compromised brain barrier integrity in aged APP/PS1 mice.

- Demographic table has control and case columns and in control there is AD (which should be dementia) and MCI. The use of AD biomarker negative cognitively impaired controls is helpful and should be addressed in the text and also findings in this group clearly discussed.

RE: We have changed AD to AD-dementia in the revision.

- a deeper characterization of cells with scRNAseq would be a great addition

RE: We are actively seeking additional grant funding for a deeper cellular characterization using single-cell RNA sequencing (scRNAseq).

- A graphical abstract is needed

RE: Thank you for recommending clearer data visualization.

Minor comments
- YO beads should be spelled out

RE: We have spelled out YO beads as Yellow Orange beads in the revision.

- Line 62: we suggest changing “AD’s underlying pathogenic mechanisms” to “AD’s possible underlying pathogenic mechanisms” since the modest success of the latest anti-amyloid treatments have created disputes about the amyloid hypothesis.

RE: We have modified the text accordingly.

- Minor typographical error in line 63: “originated” à “originates”.

RE: We have corrected the text accordingly.

- Some sentences and paragraphs should be re-written for clarity e.g.: line 230—which group? Line 232 what is meant by “expression level”? paragraph starting line 263 what’s the difference with prior paragraph?

RE: We have modified the text accordingly.

- Line 283, why not provide AUC, similar to what follows?

RE: Thank you for your suggestion. We have already presented a ROC curve utilizing blood monocyte surface Aβ biomarkers to predict brain Aβ-PET burden. It would be redundant to employ these biomarkers for predicting cognitive function, especially considering the accessibility of cognitive tests compared to brain Aβ-PET tests.

- Line 294—not clear what model is being considered?

RE: We have modified the text accordingly.

References

1. Honarpisheh P et al. Potential caveats of putative microglia-specific markers for assessment of age-related cerebrovascular neuroinflammation. J Neuroinflammation. 2020 Dec 1;17(1):366. doi: 10.1186/s12974-020-02019-5.
2. Masliah E et al. Immunoreactivity of CD45, a protein phosphotyrosine phosphatase, in Alzheimer's disease. Acta Neuropathol. 1991;83(1):12-20. doi: 10.1007/BF00294425.

3. Chen SH et al. Amyloid-beta uptake by blood monocytes is reduced with ageing and Alzheimer's disease. Transl Psychiatry. 2020 Dec 8;10(1):423. doi: 10.1038/s41398-020-01113-9.
4. Yan P et al. Peripheral monocyte-derived cells counter amyloid plaque pathogenesis in a mouse model of Alzheimer's disease. J Clin Invest. 2022 Jun 1;132(11):e152565. doi: 10.1172/JCI152565.
5. Muñoz-Castro C et al. Monocyte-derived cells invade brain parenchyma and amyloid plaques in human Alzheimer's disease hippocampus. Acta Neuropathol Commun. 2023 Feb 28;11(1):31. doi: 10.1186/s40478-023-01530-z. PMID: 36855152; PMCID: PMC9976401.

Reviewer #4 (Remarks to the Author):

In this manuscript, Huang and co-workers examine the correlation between Aβ++ monocytes in blood and CSF, brain Aβ burden by PET and cognitive decline in MCI and AD. They report a negative correlation between monocyte carriage of Aβ and cognitive decline/brain amyloid β accumulation in AD. They also propose that fluorophore-tagged PBMCs can be detected in deep cervical lymph nodes and blood after intraventricular injection in APP/PS1 mice. The topic is interesting but the results presented in this manuscript are often difficult to interpret and no conclusive evidence for clearance of Aβ by monocytes is provided.

Major points:

• The authors need to demonstrate which epitope(s) the antibody W0-2 binds to on monocytes and demonstrate specificity.

RE: The specific epitopes to which the W0-2 antibody binds on Aβ have been reported in a prior study (Ida et al. 1996). To address you and other Reviewer’s concern, we have incorporated a sketch map illustrating the antibody binding domains in the revision.

Reference:

Ida N, Hartmann T, Pantel J, Schröder J, Zerfass R, Förstl H, Sandbrink R, Masters CL, Beyreuther K. Analysis of heterogeneous A4 peptides in human cerebrospinal fluid and blood

by a newly developed sensitive Western blot assay. J Biol Chem. 1996 Sep 13;271(37):22908-14. doi: 10.1074/jbc.271.37.22908. PMID: 8798471.

• The authors should characterize the small fraction of Aβ++ intermediate monocytes more carefully on a transcriptomic level.

RE: In alignment with Reviewer #3's feedback, we are committed to conducting single-cell RNA sequencing (scRNAseq) on CSF monocytes to provide a more in-depth transcriptomic characterization.

• The correlations between Aβ++ monocytes in blood and CSF, brain Aβ burden by PET and cognitive decline in MCI and AD in Fig. 6 are quite weak.

RE: Human studies usually have greater inter-individual variations. Despite the weaker correlations, the obtained P values with statistical significance are meaningful and contribute to the overall understanding of the relationships examined.

• The sensitivity of the base model is low and statistical significance is not reached (Fig. 6).

RE: The basic panel relied solely on demographic characteristics (i.e., age, sex, years of education and APOE genotype). Comparing with the basic panel, the additional 4 biomarkers in the new panel predicted accurately for subjects with high brain Aβ-PET burden.

• Abstract: Strange to call the cells “Aβ expressing monocytes” (line 34) if the point is that they have bound to it/consumed it for clearance purposes, rather than producing it themselves.

RE: We have incorporated your suggestion in the revision.

• The number of independent biological replicates is extremely unclear throughout. This information should be provided in figure legends and made clearer in figures. Few convincing quantitative analyses have been performed.

o Figure 1: Very little information given about N numbers. Good to show representative plots (if these are representative) but why has no quantification been performed on anything? Where is quantification to support the claim that "the majority of Aβ resided on the surface of CD14+CD16+ monocytes"? The surface Aβ staining doesn't look like surface staining.

RE: We have incorporated N numbers in the revision.

o Figure 2: It is not clear what stats were performed and how significance was determined. No information in figure legend. The plots you are finding significance for (e.g. D, F, G) have values running off the graph (as discussed further below) so how can you accurately report on their fluorescent values to perform comparisons?

RE: We have incorporated appropriate stats in the revision.

o N number for supplementary figure 3 unclear. How many replicates is this based on? Why are the highest expressing CD14+ cells not included in the gates in b? Same issue about lack of clarity for N numbers throughout supplementary figures 1-5. Statistics in supplementary figure 3?

RE: We have incorporated N numbers and appropriate stats in the revision.

o In cases where data values for individual replicates are available (e.g. in supplementary table 1 related to figure 7), plots from the most extreme cases appear to have been used, rather than representative plots (as discussed further below).

RE: Those figures are representative for better visualization. The cell counts can be found in Suppl. Table 1.

• In many cases, flow scale problems suggest incorrect voltage settings were used. All fluorophore signals should fall within the plot.

RE: The FACS template was built to accommodate most human blood samples, and these settings align with those employed in a previous publication (Huang et al., 2023). It's worth

noting that monocyte surface Aβ is not a receptor, and highly phagocytic cells may exhibit extremely high Aβ on their surface. Our FACSCalibur (BD) has a limited power range up to 10^4, in contrast to 10^7 of more advanced models like FACSArial III (BD). We have checked the Aβ-binding monocytes during FACS sorting using an FACSArial III. It seemed those out-of-range cells could be fitted within the scale between 10^4 and 10^5.

o For examples, Supplementary Fig 1: Why are the cells with the highest signal off the scale (in the green boxes)? It makes it difficult to interpret.

RE: See above.

o Figure 1a, 1c and Figure 2: Again, flow scale problems suggesting incorrect voltage settings used. All fluorophore signals should be within the plot.

RE: See above.

o Figure 4: Data running off plots again so very difficult to interpret the stats.

• Figure 7: Why are no statistical comparisons or quantitative results provided from the different mice? Based on the supplementary table provided, the authors have decided to show the plots from the mouse with the highest number of "migrating" cells in the figure (i.e. 42 cells out of 666,000 in the lymph node), rather than a more representative plot. In general, the numbers of cells detected are extremely low – one of the samples the authors are using to claim for peripheral migration of the injected cells to lymph nodes includes a mouse with only 1 positive cell out of 817,000 events – and the results are similar for the other samples. The same is true for the blood data (e.g. 1 cell out of 1 million events for sample 9). This is not convincing and one could just as easily imagine someone making the exact opposite conclusion (i.e. lack of migration) based on these data.

o Related to this, the authors claim in the discussion that "Over a two-day period, an average of 11 cells migrated into peripheral blood and dcLNs, indicating that monocytes have the capacity to exit the CNS and re-enter the periphery (Fig. 7)". Where is this average of 11 coming from? Based on the supplementary table provided, the average for blood is 3.8 cells and the average for dcLNs is 7.7 cells. This is another example of extremely puzzling reporting.

RE: This is a qualitative study aimed at proposing a new hypothesis rather than a quantitative analysis. The lower numbers of CFSE+ cell counts found in peripheral or lymph node, even as low as one, still represent the possibility of leukocyte in CSF migration back to home. In the context of such low-frequency situations, calculating averages may not offer significant insights. The 11 cells represent the average total of detected cells in both blood and dcLNs per mouse, calculated as 3.8 + 7.7 = 11.5.

• Several points in the discussion are puzzling.
o For example, what do you mean when you say "Our research, in line with others, discovered the presence of peripheral leukocytes in the CNS"?
o The following claims are too strong, as the functional importance of these Aβ- carrying cells is not shown and is based on correlations "Our study underscores the vital role of peripheral monocytes in the pathogenesis of AD, serving as essential players in the phagocytosis and transportation of Aβ peptides between the CNS and periphery"
• In lines 127-129: The authors state "Similarly, in CSF, the majority of CD45+CD14+CD16+ monocytes also express CD68 and Trem2 (Fig. 1c-d)". You have analysed extremely few cells from the CSF (Figure 1d). And why do you call these monocytes if they have macrophage phenotypes? CD14 and CD16 are also expressed by certain CNS macrophages, such as some reactive and plaque-associated human microglia (see, for example, PMID: 8227309 and PMID: 26286145).

RE: We have incorporated your suggestions in the revision. Those CSF monocytes were macrophage-like and microglia-like.

Minor points:

o No legend is provided for supplementary figure 5.

RE: We have incorporated a legend in the revision.

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

RE: We are grateful for your thorough review of our work.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed all comments. This is a very interesting study.

Reviewer #2 (Remarks to the Author):

I want to compliment the team for the end result, their efforts and improvements made in the readibility.
They have sufficiently addressed my concerns.
There is one inclarity: about the phagocytosis system, section 2.3, they mentioned that the developed experimental system incorporated AF647-conjugated Aducanumab to visualize surface-bound oAβ1-42, but I do not see results pertaining to this tracking. Did it not work, or did I miss something?

Moreover, one important basis of the proof that peripheral monocytes enter the brain is based on their expression of CD14/CD16, isn't? This could be re-iterated or stressed more to convey the message.

I am impressed by the work done!

Reviewer #3 (Remarks to the Author):

The current version of the manuscript is improved from the initial. We were particularly interested by the newly added findings suggesting a U-shaped relationship between Aβ++ monocyte MFI and PET-amyloid, and the monocyte subgroup similarities of the CU+ve group with MCI and AD groups. We were also pleased to see a high percentage of CSF monocytes expressing the peripheral marker CD163. However, additional work is still needed to support the main results of this study and clarify some points.

Major comments
- The graphical abstract focuses on brain anatomy rather than the results of the study. Panel D is the only one presenting, albeit partially, some of the study's main findings.
- Table 1 presents groups based on amyloid positivity, however only one analysis implements this distinction, the majority using the CU/MCI/AD grouping. It would be better to present Table 1 grouped in this way and include info on Aβ PET positivity as a row.
- Results, Paragraph 3.5: "Subsequently, a panel comprising four biomarkers … outperformed other examined biomarker combinations". It would be of interest to the reader to compare the predictive ability of single biomarkers with the final panel. We suggest the addition of a Table in supplementary materials showing AUCs for single markers and different combinations.
- Results, Paragraph 3.6: The phrasing initially seems to suggest that there were statistically significant differences between CU-ve and CU+ve groups. It should be noted at the beginning of the paragraph that there were no significant differences between the two groups, and the differences should be rather described as observations, or trends if the p-value approaches significance.
- Supplementary table 2, Part 1: it seems that the numbers for % and MFI are switched.
- Results, paragraph 4: The authors performed an experiment with beads that strengthened their argument. However, contrary to our suggestion, they did not show infiltration of peripheral intermediate monocytes in the brain, which would be a crucial validation experiment. Tracking of peripheral monocyte infiltration in the brain has previously been demonstrated in mice (Ref 1) and

therefore is attainable.
- Discussion, implications for AD diagnostics: The authors suggest that Aβ-carrying monocytes could be a useful biomarker for AD. However, this is not supported by the data. As we had pointed in the previous review round, a large proportion of CU participants had levels of Aβ+ monocytes similar to MCI and AD patients, however this is not elaborated upon. Furthermore, the model including the monocyte variables outperformed the basic model by a small margin (AUC 0.81 vs 0.87), and no changes were found between MCI and AD participants. Why would the use monocytes be preferred if other sensitive and well-characterized blood based biomarkers already exist?
- Discussion, implications for AD diagnostics: The following sentences are not directly relevant to the findings of the study – "In a study involving 252 participants from 483 AIBL, AD patients exhibited a 16% reduction in plasma Aβ1-42 compared to healthy controls. The plasma Aβ1-42/Aβ1-40 ratio, phosphorylated-tau181, glial fibrillary acidic protein, and neurofilament light were also evaluated as potential blood biomarkers for AD".
- The authors did not discuss previous work pertaining to their findings in the Discussion (Ref 1-3), despite confirming the inclusion of said references in the rebuttal. This is important as it connects their evidence to previous knowledge and enables the reader to understand the novelty of their findings.

Minor comments
- Abbreviations should be spelled out in the legend of tables and figures.
- Results, Paragraph 4: Figure number should be 6 instead of 5. Furthermore, in line 401 it should be Fig. 6h instead of g.

References
1. Yan P et al. Peripheral monocyte-derived cells counter amyloid plaque pathogenesis in a mouse model of Alzheimer's disease. J Clin Invest. 2022 Jun 1;132(11):e152565. doi: 10.1172/JCI152565.
2. Chen SH et al. Amyloid-beta uptake by blood monocytes is reduced with ageing and Alzheimer's disease. Transl Psychiatry. 2020 Dec 8;10(1):423. doi: 10.1038/s41398-020- 01113-9.
3. Muñoz-Castro C et al. Monocyte-derived cells invade brain parenchyma and amyloid plaques in human Alzheimer's disease hippocampus. Acta Neuropathol Commun. 2023 Feb 28;11(1):31. doi: 10.1186/s40478-023-01530-z. PMID: 36855152; PMCID: PMC9976401.

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I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Point-by-point response to the reviewers' comments

REVIEWERS' COMMENTS

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I want to compliment the team for the end result, their efforts and improvements made in the readibility.
They have sufficiently addressed my concerns.
There is one inclarity: about the phagocytosis system, section 2.3, they mentioned that the developed experimental system incorporated AF647-conjugated Aducanumab to visualize surface-bound oAβ1-42, but I do not see results pertaining to this tracking. Did it not work, or did I miss something?

RE: The AF647-conjugated Aducanumab to visualize surface-bound oAβ1-42 worked as shown in Suppl. Fig. 6a-c (Y axis FL4::AF647-Adu). The abbreviation 'Adu' for Aducanumab has been included in the figure legend.

Moreover, one important basis of the proof that peripheral monocytes enter the brain is based on their expression of CD14/CD16, isn't? This could be re-iterated or stressed more to convey the message.

RE: Indeed, the expression of CD14/CD16 by peripheral monocytes is a crucial basis for proving their entry into the brain. We have reiterated this point in Chapter 4 of the Results section and in Suppl. Fig. 11 to ensure the message is clearly conveyed.

I am impressed by the work done!

Reviewer #3 (Remarks to the Author):

The current version of the manuscript is improved from the initial. We were particularly interested by the newly added findings suggesting a U-shaped relationship between Aβ++ monocyte MFI and PET-amyloid, and the monocyte subgroup similarities of the CU+ve group with MCI and AD groups. We were also pleased to see a high percentage of CSF monocytes expressing the peripheral marker CD163. However, additional work is still needed to support the main results of this study and clarify some points.

Major comments
- The graphical abstract focuses on brain anatomy rather than the results of the study. Panel D is the only one presenting, albeit partially, some of the study's main findings.

RE: Nature Communications do not allow a graphic abstract. Therefore, it has been removed as requested.

- Table 1 presents groups based on amyloid positivity, however only one analysis implements this distinction, the majority using the CU/MCI/AD grouping. It would be better to present Table 1 grouped in this way and include info on Aβ PET positivity as a row.

RE: As the main purpose of Methods Chapter 3, 'A Potential Biomarker for AD,' is to predict brain Aβ, Table 1 is designed to clearly indicate the number of cases and controls in the study. This is noted in the footnote: 'Stratified by brain Aβ-PET burden. The demographic table was used for binary logistic regression, leading to the reclassification of the study cohort into two groups: ⩽25 CL and >25 CL, rather than using categories such as AD dementia, MCI, and CU.' This stratification aligns with the chapter's focus on predicting brain Aβ.

- Results, Paragraph 3.5: “Subsequently, a panel comprising four biomarkers … outperformed other examined biomarker combinations”. It would be of interest to the reader to compare the predictive ability of single biomarkers with the final panel. We suggest the addition of a Table in supplementary materials showing AUCs for single markers and different combinations.

RE: We have added Supplementary Tables 3 and 4 to provide the AUCs for single markers and different combinations. This additional information should help readers compare the predictive abilities more effectively.

- Results, Paragraph 3.6: The phrasing initially seems to suggest that there were statistically significant differences between CU-ve and CU+ve groups. It should be noted at the beginning of the paragraph that there were no significant differences between the two groups, and the differences should be rather described as observations, or trends if the p-value approaches significance.

RE: We have modified the paragraph to clarify that there were no statistically significant differences between the CU-ve and CU+ve groups. Instead, the differences are described as observations or trends. Here is the revised text:

“Although there were no statistically significant differences between CU-ve and CU+ve groups, we observed that many CU participants exhibited low surface Aβ levels overlapping with the MCI and AD-dementia groups. We compared CU individuals based on brain Aβ-PET status (CU-ve: ⩽25 CL, n=49; CU+ve: >25 CL, n=29), MCI+ve (n=27), and AD-dementia (n=36), excluding those with MCI-ve (n=9) from the comparison. The results indicated that CU+ve individuals had a percentage of $A\beta^{++}$ monocytes lower than that of CU-ve individuals and similar to that of MCI+ve/AD-dementia across all three monocyte subsets (Suppl. Table 2).”

- Supplementary table 2, Part 1: it seems that the numbers for % and MFI are switched.

RE: Thank you for pointing that out. We have corrected the numbers for % and MFI in Supplementary Table 2, Part 1.

- Results, paragraph 4: The authors performed an experiment with beads that strengthened their argument. However, contrary to our suggestion, they did not show infiltration of peripheral intermediate monocytes in the brain, which would be a crucial validation experiment. Tracking of peripheral monocyte infiltration in the brain has previously been demonstrated in mice (Ref 1) and therefore is attainable.

RE: Thank you for your feedback. We have shown the infiltration of peripheral intermediate monocytes in the human CSF and brain in Fig. 6 and Suppl. Fig. 11. There are other studies similar to Ref 1, such as Baruch et al. (2016), which demonstrated PD-1 immune checkpoint blockade reducing pathology and improving memory in mouse models of Alzheimer's disease (Nat Med. 2016;22(2):135-7). Therefore, in this paper, we focused on whether these peripheral monocytes can return to the periphery. We have added these two references. Thank you for your suggestion.

- Discussion, implications for AD diagnostics: The authors suggest that Aβ-carrying monocytes could be a useful biomarker for AD. However, this is not supported by the data. As we had pointed in the previous review round, a large proportion of CU participants had levels of Aβ+ monocytes similar to MCI and AD patients, however this is not elaborated upon. Furthermore, the model including the monocyte variables outperformed the basic model by a small margin (AUC 0.81 vs 0.87), and no changes were found between MCI and AD participants. Why would the use monocytes be preferred if other sensitive and well-characterized blood based biomarkers already exist?

RE: Thank you for your feedback. We have previously discovered four blood-based biomarkers for AD diagnosis (Huang et al., "Leukocyte surface biomarkers implicate deficits of innate immunity in sporadic Alzheimer's disease," Alzheimers Dement. 2023 May;19(5):2084-2094. doi: 10.1002/alz.12813. Epub 2022 Nov 9. PMID: 36349985; PMCID: PMC10166765).

While we acknowledge that a large proportion of CU participants had levels of Aβ+ monocytes similar to MCI and AD patients, our model, which includes monocyte variables, did show improved performance (AUC 0.87) over the basic model (AUC 0.81). Although the margin is small, this improvement suggests that Aβ-carrying monocytes have potential as a biomarker for AD.

We remain open to validating our findings further to establish Aβ-carrying monocytes as a useful biomarker for AD in the future. The proposal is a potential development, given the benefits of blood-based biomarkers: they are cheaper and non-invasive compared to amyloid-PET.

- Discussion, implications for AD diagnostics: The following sentences are not directly relevant to the findings of the study – "In a study involving 252 participants from 483 AIBL, AD patients exhibited a 16% reduction in plasma Aβ1-42 compared to healthy controls. The plasma Aβ1-42/Aβ1-40 ratio, phosphorylated-tau181, glial fibrillary acidic protein, and neurofilament light were also evaluated as potential blood biomarkers for AD".

RE: You are right, these sentences have no direct relevance. We have modified the paragraph to clarify that. Here is the revised text:

"In a study involving 252 participants from AIBL, AD patients exhibited a 16% reduction in plasma Aβ1-42 compared to healthy controls. The plasma Aβ1-42/Aβ1-40 ratio, phosphorylated-tau181, glial fibrillary acidic protein, and neurofilament light were also evaluated as potential blood biomarkers for AD. In addition to plasma Aβ diagnostics for AD, our findings highlight the potential use of blood cell-based Aβ diagnostics. The correlation between the reduction in Aβ-carrying monocytes in AD patients and their association with brain Aβ burden and cognitive decline underscores the clinical relevance of our study, emphasizing the potential for these markers to enhance AD diagnosis and prognosis."

- The authors did not discuss previous work pertaining to their findings in the Discussion (Ref 1-3), despite confirming the inclusion of said references in the rebuttal. This is important as it connects their evidence to previous knowledge and enables the reader to understand the novelty of their findings.

RE: As we have demonstrated through similar literature, such as Baruch et al. (2016), we can only include a limited selection of references in each area covered by this study. For example, Ref 1 (Yan et al.) supports the infiltration of peripheral monocytes in AD mouse brain, and Ref 3 (Muñoz-Castro et al.) expands on the findings discussed in Results Chapter 4 of our study. However, we opted not to include Ref 2 (Chen et al.) due to reservations about the methods used in that study.

Minor comments
- Abbreviations should be spelled out in the legend of tables and figures.

RE: We have included a list of abbreviations.

- Results, Paragraph 4: Figure number should be 6 instead of 5. Furthermore, in line 401 it should be Fig. 6h instead of g.

RE: Thank you for your feedback. We have made the corrections accordingly.

References
1. Yan P et al. Peripheral monocyte-derived cells counter amyloid plaque pathogenesis in a mouse model of Alzheimer's disease. J Clin Invest. 2022 Jun 1;132(11):e152565. doi: 10.1172/JCI152565.
2. Chen SH et al. Amyloid-beta uptake by blood monocytes is reduced with ageing and Alzheimer's disease. Transl Psychiatry. 2020 Dec 8;10(1):423. doi: 10.1038/s41398-020- 01113-9.
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Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.