nature portfolio

Peer Review File

Therapeutic efficacy of intracerebral hematopoietic stem cell gene therapy in an Alzheimer's disease mouse model

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The therapeutic potential of engineered HSPC transplantation has been implicated in monogenic neurodegenerative diseases. With pre-conditioning like alkylating agent busulfan (BU), HSPCs could infuse the CNS and become microglia-like cells. To enhance the engraftment and the maturation of transplanted HSPCs into the CNS, Milazzo et al. compared the engraftment efficiency of HSPC delivered through three different routes after BU-based myeloablation - intracerebral ventricles (ICV), intrathecal lumbar (ITL), and intravenous (IV) injection. The authors also analyzed the phenotypes of the microglia-like cells transplanted via different routes using immunohistochemistry, RT-PCR and RNA scope, and make the following major claims:
(1) CNS-administrated delivery (through both ICV and ITL) of HSPCs displayed better engraftment than IV-delivered HSPCs in a competitive setting
(2) ITL delivery resulted in decent chimerism in the BM
(3) The routes of HSPC administration led to differential distribution patterns of engrafted microglia-like cells
(4) Intra-CNS-delivered HSPCs favor the maturation towards ramified microglia-like cells and exhibited higher expression of homeostatic microglial cells
Lastly, the authors also applied their HSPC gene therapy in an AD model and showed beneficial effects via ICV delivery of HSPCs expressing hTREM2. This study provides a comprehensive understanding of different HSPC transplantation strategies into the CNS. In addition, the authors tested the transplantation platforms and showed that they have great therapeutic potential in treating neurodegenerative conditions. The authors also tried to provide more cellular and molecular characteristic of how the microglia-like cells delivered from various routs are different. However, there remain some concerns regarding the experimental approaches and interpretation. I'd like to provide some suggestions on refining the paper.

Major concerns:
(1) The statistical analysis in Figure 1 is quite confusing, why were the authors labeling the groups with $p<0.5$ instead of 0.05? Could the authors re-organize the graph and labeling to make the conclusion more comprehensive?
(2) Does TAμ and μ have any differential distribution across different brain regions? If so, will the conclusion from Figure 4 largely depend on the regional distribution of engulfed microglia (cell-extrinsic effect) instead of a route-dependent cell-intrinsic effect? Especially because the Figure 4 is done at early time point after the transplantation (45 days), could the authors look into later time point (e.g. 90 days after transplantation) and try to address the question further?
(3) Could the authors discuss through which routes do the HSPCs get into the CNS, and do they differ dependent on the routes of injection? For example, does the cells injection via ICV mainly entering through the choroid plexus, and whether this might explain the distribution pattern of ICV-delivered cells?
(4) How helpful is it to differentiate TAμ and μ? The ICV-delivered TAμ and μ do not obtain

differential expression of homeostatic genes but the two subsets remain (Figure 4D). How instructive is it to use TAμ and μ subsets to infer the current transplantation data?
(5) The Figure 3D and Figure 3E are confusing, why don't the authors just show and compare the Sholl analysis (plot by Number of points/Distance from the cell body) of microglia-like cells transplanted from different routes?
(6) It is interesting that the authors observed differential expression of microglial homeostatic genes in ICV-delivered microglia-like cells versus the other routes. Could the authors perform bulk RNA-seq on the TAμ and μ cells from different routes? That might give the authors more insights on what drives the differences?
(7) For Figure 5, could the authors verify the hTREM2 expression in the ICV-hTREM2 expression group via IHC?

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(2) Please label the probes (Tmem119 and Tgfbr) in Figure 4F
(3) Please indicate more clearly how long after the transplantation does the authors performed the morphological analysis in the main text and figure legend.
(4) In line 258, "through BAC-mediated transgenesis showed encouraging results in ameliorating the scavenging function", is the ameliorating supposed to be "enhancing"?

Reviewer #2 (Remarks to the Author):

NCOMMS-392072

Transplantation of engineered hematopoietic stem and progenitor cells in the CNS uniquely shapes their engraftment, distribution and differentiation, and benefits Alzheimer's disease in mice.

Milazzo et al.

Transplantation of genetically modified hematopoietic cells can provide long-term therapeutic benefits for young patients with rare genetic diseases whose symptoms are caused by dysfunctional enzymes and the associated accumulation of the enzyme's substrates. The utility of the therapeutic approach for treating older patients with non-genetic common diseases, such as Alzheimer's disease, remains unclear.

Here the authors build on their earlier work to describe an excellent series of experiments to compare different routes of administration and different sources of donor cells that engraft the wildtype mouse brain over time. The authors then move to the heavy genetic load of the 5x FAD mouse (an early onset model of amyloidopathy) to evaluate a cell and gene therapy approach to

improve behavior and pathology in the brain after intracerebroventricular administration of TREM2 overexpressing hematopoietic cells.

Overall, the data presented regarding the timelines of engraftment in the brain, spinal cord and bone marrow using different routes of administration and donor cell types is important and robust. However, major points regarding the state of the engrafted microglia and the work in the disease model are weaknesses that need to be addressed by the authors to significantly improve the manuscript.

Major points

1. The route of administration appears to influence the morphological complexity and biochemistry of the engrafted cells in the brain. Given that direct administration of cells to the brain can cause tissue damage that modulates microglial gene expression and morphology, can the authors help the reader to interpret the data by including parallel analyses of the untransplanted, brain resident microglia to control for any such effect of tissue damage.

2. Clinically, conditioning is associated with neuronal damage that is controlled by anti-convulsants such as levetiracetam. The authors describe the use of diazepam in the 5x FAD mice but not the wildtype mice. Given this difference in transplantation protocol between the route of administration studies and the 5xFAD work, can the authors describe the dose of diazepam administered. Perhaps more importantly, it remains unclear whether neuronal damage due to conditioning is a primary driver of myeloid cell engraftment in the brain. Can the authors report the extent of neuronal damage across epileptogenic neural circuits, even if the damage is insufficient to cause seizures in all animals?

3. While TREM2 overexpression does not appear to effect hematopoietic cell engraftment of the brain, the influences of mutant APP and mutant PS1 overexpression in the 5xFAD mice upon brain engraftment is relevant to understand the proposed treatment effects. Can the authors supplement the brain VCN data by providing a detailed evaluation of cell engraftment efficiency in the 5x FAD mouse brain using assays described in the earlier work determining the route of administration? Such direct comparisons between models and platforms will help the reader to understand the role of brain engraftment in the amyloidopathy model.

4. Engrafted cells reside in both the blood and the brain but the authors suggest that efficacy is derived from parenchymal engraftment. Unfortunately, the authors do not typically perfuse the brain to minimize the confounding interpretations of engrafted cells in the blood. The manuscript will be significantly improved when the authors report data from mice with targeted perfusion of the brain with heparinized saline and formalin would help the reader to focus on brain-specific analyses of engraftment rather than the presented mixture of brain parenchyma and blood signals due non-targeted tissue preparation. The current lack of data separating these tissue compartments is a significant methodological deficit in the current manuscript.

5. The authors use a lentivirus to overexpress human TREM2 in 5x FAD mice. Given the poor amino acid sequence similarity between mouse and human TREM2, the authors need to evaluate whether

immunogenicity from the transgene expression contributes to the phenotype in the transgenic mice.

6. The manuscript does not report an untransplanted 5xFAD mouse group as a control for pathobiology and behavioral changes in the authors' experimental conditions. Historical references to the phenotypes are insufficient to help the reader to understand if conditioning, transplantation and TREM2 overexpression improves the behavior and/or the pathobiology of the 5xFAD mouse. This is a significant limitation of the study that needs to be addressed by the authors.

7. The authors declare in the reporting summary that they articulate the exact sizes of each experimental group. However, the manuscript and supplemental material does not clarify any group sizes. The authors need to help the reader to understand the strength of any statistical associations reported by explicitly stating the group sizes for each statistical analysis presented.

8. The authors mention that mice were dosed but did not show engraftment were excluded from the experimental groups. Can the authors state the total numbers of mice that were dosed and the total numbers of mice that demonstrated engraftment so that the reader can understand the reproducibility of the therapeutic approach?

Minor points

1. The authors rather surprisingly use dollar signs to represent statistical significance, which seems inappropriate. Can the authors use a different character to represent significant associations while avoiding the graphical representation linking money to statistical significance?

2. Can the authors provide the pre-transplantation vector copy numbers of the cells administered to each experimental group?

POINT-BY-POINT RESPONSE TO THE REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

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Major concerns:

(1) The statistical analysis in Figure 1 is quite confusing, why were the authors labeling the groups with $p<0.5$ instead of 0.05? Could the authors re-organize the graph and labeling to make the conclusion more comprehensive?

Response: We apologize to the Reviewer for this. As per his/her suggestion, we modified the labeling for statistical significance in Figure 1, leaving in the main figure only the labels referring to the comparison between each of the combinatorial-transplant groups and the standard IV-only reference group. Statistical data referring to the other comparisons have been included in the New Table 1 and 2. Suppl. Fig. 1 has been modified consistently and the statistics are included in the New Supplementary Table 1.

(2) Does TAμ and μ have any differential distribution across different brain regions? If so, will the conclusion from Figure 4 largely depend on the regional distribution of engulfed microglia (cell-extrinsic effect) instead of a route-dependent cell-intrinsic effect? Especially because the Figure 4 is done at early time point after the transplantation (45 days), could the authors look into later time point (e.g. 90 days after transplantation) and try to address the question further?

Response: Transplant progeny cells showing the μ and TAμ phenotypes are present in the repopulated regions of the recipients' brain with no region-specific distribution. They rather have a differential frequency over time in the post-transplant CNS. Indeed, at the shorter time points of post-transplant analysis TAμ cells are more abundant than μ. At longer time points, donor-derived TAμ would progressively reduce in frequency and then disappear in favor of μ, consistently with a stepwise maturation process reminiscent of microglia maturation in early post-natal life. For this reason, we selected a relatively early time point of analysis (45 days post-transplant) that could allow us depicting differences in the two cell populations and obtaining relevant insight on the microglia reconstitution

process. Indeed, at this time point we observed the greatest difference between TAμ and μ frequency in the tested transplant conditions. Of note, the morphological and transcriptional characteristics of TAμ cells and the timing of their appearance in the CNS of wild type transplant recipient mice support the definition of them being immature, rather than "engulfed", cells. These considerations were included in the text (Results section).

(3) Could the authors discuss through which routes do the HSPCs get into the CNS, and do they differ dependent on the routes of injection? For example, does the cells injection via ICV mainly entering through the choroid plexus, and whether this might explain the distribution pattern of ICV-delivered cells?

Response: Understanding the mechanisms and timing of HSPC/their progeny penetration into the CNS parenchyma post-transplant would be of great relevance to increase the success of a stem cell-based treatment and to possibly reduce the number of the administered cells. For this reason, it is object of intense investigation by many groups. Our group in the past conducted some experiments to monitor the fate of HSPCs shortly after transplant based on the combined use of magnetic resonance imaging (MRI), flow cytometry and immunohistology (Capotondo et al 2012). To this scope, before transplantation in myeloablated recipients via IV infusion, the cells were transiently labelled with SPIO-based contrast media and genetically modified with LVs to express a fluorescent reporter. MRI demonstrated multiple hypointense spots within the brain of the transplanted animals as early as 24 hours post-injection, suggesting an early seeding of transplanted cells that were then identified also by flow cytometry and histology in the brain parenchyma of the perfused animals. These data were also confirmed by functional data of serial transplantation showing the tendency of the infused cells to shortly migrate into the CNS. Interestingly, at these very early time points post-transplant, the transplanted cells were identified in periventricular regions, where also ICV-injected cells are found early post-transplant. These data allow us to hypothesize that brain penetration at the choroid plexus and trans-ependymal migration could be the most likely routes used by HSPCs to enter and distribute within the CNS. Of course, the ITL and ICV administration have the advantage of shortening the path to the target location, thus reducing the unwanted dissipation of transferred stem cells to other organs. Furthermore, it is logical to assume that IV and intra-CNS injected cells may use different pathways and respond to different stimuli to egress blood vessels or ependyma, respectively. To better clarify these aspects, we implemented some of these comments more robustly into the Discussion section of the manuscript.

(4) How helpful is it to differentiate TAμ and μ? The ICV-delivered TAμ and μ do not obtain differential expression of homeostatic genes but the two subsets remain (Figure 4D). How instructive is it to use TAμ and μ subsets to infer the current transplantation data?

Response: As discussed above, TAμ and μ subsets represent sequential stages of maturation of newly generated, transplant-derived myeloid cells sharing phenotypic features of immature myeloid cells retrieved in the brain of neonates (TAμ) at the early time-points post-transplant, then progressively maturing into cells functionally reminiscent of adult naïve microglia (μ). The more the gene expression signature of these populations resembles that of adult microglia, the more they progress, as per our experimental hypothesis, in their maturation. The finding that TAμ and μ in the ICV setting express microglia homeostatic genes in the range of reference adult microglia, differently than TAμ (and μ) cells in the IV and ITL settings, supports the concept that the former underwent a substantial maturation as compared to latter. The IV setting is associated to a slower/less efficient maturation of HSCT-derived myeloid cells as compared to ICV, and the ITL one results in an intermediate maturation where only μ have reached a maturation sufficient to express the microglia homeostatic genes in the range of reference adult microglia. Thus, in our opinion, the differential analysis of TAμ and μ subsets increases our ability to capture information about the process of differentiation and maturation of donor derived myeloid cells in the CNS towards functionally equivalent microglia cells.

(5) The Figure 3D and Figure 3E are confusing, why don't the authors just show and compare the Sholl analysis (plot by Number of points/Distance from the cell body) of microglia-like cells transplanted from different routes?

Response: Figure 3D graphically presents the distribution of donor derived microglia like cells depending on their complexity and cell harboring. Figure 3E represents the quantification of these data allowing to appreciate the cell

frequency retrieved in the 4 quadrants. If the reviewer finds it redundant, we are willing to follow the suggestion to retain in the manuscript only Figure 3E.

(6) It is interesting that the authors observed differential expression of microglial homeostatic genes in ICV-delivered microglia-like cells versus the other routes. Could the authors perform bulk RNA-seq on the TAμ and μ cells from different routes? That might give the authors more insights on what drives the differences?

Response: The characterization of μ and TAμ populations was the object of a previous work in which the transcriptomic features of donor-derived brain-associated myeloid transplant progeny cells were deeply analyzed and compared with microglia cells retrieved from control naïve mice at different development stages (Capotondo , Milazzo et al., 2017). Gene set enrichment analysis on RNA-seq data identified a pattern of genes differentially regulated in post-transplant myeloid subpopulations, providing indications about the biological process enriched in each. In particular, newly formed cells after transplant, i.e. TAμ, were characterized by up-regulation of pathways related to neuronal processes, including neuron migration, differentiation and regulation of synaptic plasticity, as it could be expected in myeloid cells maturing in the CNS environment. The differential expression of these pathways in μ and TAμ subsets supports the hypothesis of a different stage of maturation of μ and TAμ, in agreement with the concept that microglia acquire different functions according to their maturation state. As the aim of the current work was to confirm and compare the maturation of the progeny of the HSPC delivered via different routes, we focused on specific microglia gene-sets, assuming that a selected microglia signature would be sufficient to address this aspect.

(7) For Figure 5, could the authors verify the hTREM2 expression in the ICV-hTREM2 expression group via IHC?

Response: We evaluated the expression of hTREM2 on the brain tissue extract of ICV transplanted animals (N=6) by quantitative RT-PCR (RT-qPCR) using a SYBR Green Kit with primers and probe from Thermofisher (Catalog number: 4448892, Hs01003898_m1, Product size: 91 bp, Dye: FAM). For this gene expression analysis, we preliminary validated the hTREM2 primers on human THP1 cells, a monocytic cell line that constitutively expresses hTREM2, and on BV2 murine cells transduced with the LV_hTREM2, both used as positive controls for hTREM2 expression. All values were normalized to the housekeeping gene GAPDH. hTREM2 expression was then measured on the brain of the study animals transplanted ICV with HSPCs transduced with the therapeutic LV-hTREM2, collected at sacrifice 10 months after the transplant. Importantly, a significant correlation was observed between hTREM2 expression measured by RT-qPCR and the LV-hTREM2 content, measured as vector copy number per cell on the brain tissue of the transplanted animals ($R2 = 0.99$) (see **New Figure 5H**).

Minor concerns:

(1) In Figure 4C, the authors showed the RT-PCR result of p2ry13. Is it a typo? Or were the authors measuring p2ry13 but not p2ry12?

Response: In the present study we measured the expression of P2ry13, consistently with our previous work Capotondo, Milazzo et al., 2017). P2Y12 and P2Y13 receptors share a similar pharmacology and molecular structure (48% identical at the amino acid level; Perez-Sen et al., 2017). Microglia express P2Y13 receptor at levels almost as high as for P2Y12 (Zhang et al., 2014). Indeed, according to transcriptome data from other groups, P2ry13 is considered the second most expressed neurotransmitter receptor in microglia (Kyrargyri et al., 2019). In the brain P2ry13 is expressed predominantly by microglia, much less by oligodendrocyte precursor cells and myelinating oligodendrocytes, and not by other brain cell types (Zhang et al., 2014).

(2) Please label the probes (Tmem119 and Tgfbr) in Figure 4F.

Response: We added labeling of the probes in Figure 4F.

(3) Please indicate more clearly how long after the transplantation does the authors performed the morphological analysis in the main text and figure legend.

Response: We added this information in the main text and figure legend.

(4) In line 258, "through BAC-mediated transgenesis showed encouraging results in ameliorating the scavenging function", is the ameliorating supposed to be "enhancing"?

Response: Yes, it is.

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Overall, the data presented regarding the timelines of engraftment in the brain, spinal cord and bone marrow using different routes of administration and donor cell types is important and robust. However, major points regarding the state of the engrafted microglia and the work in the disease model are weaknesses that need to be addressed by the authors to significantly improve the manuscript.

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1. The route of administration appears to influence the morphological complexity and biochemistry of the engrafted cells in the brain. Given that direct administration of cells to the brain can cause tissue damage that modulates microglial gene expression and morphology, can the authors help the reader to interpret the data by including parallel analyses of the untransplanted, brain resident microglia to control for any such effect of tissue damage

Response: Optimized protocols are currently in use in both preclinical and clinical practice allowing safe ICV administration of drugs, proteins or cells in animals and patients (Nizzardo M et al 2017; Galvan A et al 2021; Metz T et al 2022; Cohen-Pfeffer MD et al 2017). Importantly, several clinical studies demonstrated that adverse events of intrathecal applications are rare and associated to mild and temporary side effects (Singer W 2019). According to our direct experience, stereotaxic machine-guided ICV injection can cause a transient and localized recruitment of inflammatory cells along the needle track, which normally resolves within a few days. Consistent to our experience are the findings of Granados-Duran and colleagues, who performed a detailed monitoring of rats at 2-4-12-24 hours, 2-4-7-15-30 days and at 3 and 6 months after the ICV administration of pharmacological compounds to monitor for the possible occurrence of neuroinflammation (Granados-Dural et al 2015). Importantly, the authors found that control rats, which were ICV-injected with vehicle, only showed a mild inflammatory reaction in the cortex, at the level of the needle path, that spontaneously healed within a very short time (≤2 weeks). No inflammation was observed in the parenchyma outside of the needle tract nor in the brain ventricles, meninges or in the periventricular regions at any of the time points of analysis. Of note, our morphological and gene expression analysis on the brain of ICV-injected animals was intentionally performed at 45 days post-transplant - a time point where signs of cell activation due to surgery are no longer expected. In agreement with this, we did not retrieve engulfed nor activated microglia cells in the area close to the injection site that could suggest the presence of an ongoing inflammatory process at the time of evaluation; resident microglia in the periventricular areas of the injected hemispheres appeared well ramified and with small cell bodies, reminiscent of cells in resting state. As reference control, we analyzed mature resident microglia that were included in the gene expression panels as reference adult μ.

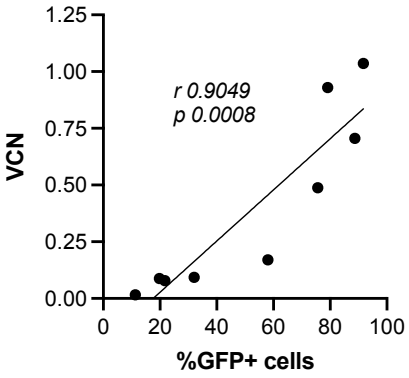
2. Clinically, conditioning is associated with neuronal damage that is controlled by anti-convulsants such as levetiracetam. The authors describe the use of diazepam in the 5x FAD mice but not the wildtype mice. Given this

difference in transplantation protocol between the route of administration studies and the 5xFAD work, can the authors describe the dose of diazepam administered. Perhaps more importantly, it remains unclear whether neuronal damage due to conditioning is a primary driver of myeloid cell engraftment in the brain. Can the authors report the extent of neuronal damage across epileptogenic neural circuits, even if the damage is insufficient to cause seizures in all animals?

Response: Busulfan chemotherapy causes crosslinking of DNA and has been shown to induce cell cycle arrest of proliferating cells. Because of this mechanism of action, and of its ability to cross the BBB (with 20% of the plasmatic active drug concentration being retrieved in the CNS), busulfan has effects on proliferating cells in the CNS, including adult neural progenitors whose neurogenesis potential is extinguished in mice treated with myeloblative busulfan-based regimes, oligodendrocyte progenitor cells (NG2) and microglia that in the same conditions exhaust their regenerative capacity. These long-term effects were shown in mice (Sailor et al. 2022) but are not paralleled by clinical observations. Instead, neurotoxicity concurrent to dosing, mostly in the form of seizures, has been reported in clinical practice with the use of busulfan, especially with high doses (Vassal G. et al 1990, Meloni G. et al 1992). For this reason, the administration of anticonvulsant prophylaxis has become common during high-dose busulfan-based conditioning regimes in patients undergoing hematopoietic cell transplant. However, no specific pathological findings have been reported as associated to these epileptic events that are anyhow confined to a very specific, transient and concurrent-with-treatment administration time window. No link between this well-known side effect of busulfan and neural damage and/or the process of donor cell engraftment has ever been established. Based on these considerations, we thus believe that identifying possible signs of busulfan-related neuronal damage at the time of treatment administration and associated to drug-induced seizures would go beyond the scope of this manuscript, which focuses its attention at a time of analysis that goes long after the time when these events could have occurred. Rather, in the attempt to prevent/limit the occurrence of seizures during busulfan administration, we administered anticonvulsants to 5xFAD mice, which could be possibly more prone to develop seizures than wild type mice (that usually do not experience seizures during high dose/myeloablative busulfan regime administration) because of their CNS disease. As detailed in the Methods section, in our study, class IV pharmaceutical grade diazepam solution (available at 5 mg/mL) was provided daily in the drinking water starting for a total of 10 days with progressive decreases in drug dose (0.25 mg/kg used for the first 8 days, then dose decreased in the two following days to 0.125mg/kg - 0.063 mg/kg and removed).

3. While TREM2 overexpression does not appear to effect hematopoietic cell engraftment of the brain, the influences of mutant APP and mutant PS1 overexpression in the 5xFAD mice upon brain engraftment is relevant to understand the proposed treatment effects. Can the authors supplement the brain VCN data by providing a detailed evaluation of cell engraftment efficiency in the 5x FAD mouse brain using assays described in the earlier work determining the route of administration? Such direct comparisons between models and platforms will help the reader to understand the role of brain engraftment in the amyloidopathy model.

Response: Flow cytometry is an important tool for evaluating donor engraftment in tissues, and in particular in the brain, but we couldn't apply it to the study of transplanted cell engraftment in 5xFAD mice. Indeed, the transgenic hTREM2 protein can't be detected by flow cytometry on the transduced cells due to the lack of specific antibodies discriminating the human from the murine isoforms. Neither we could take advantage of the CD45.1/CD45.2 allele mismatch to quantify the donor engraftment in this transplantation setting due to the unavailability of 5xFAD animals with the CD45.1 allele. Thus, we could quantify the transplanted cell engraftment only by VCN measurement.
According to our experience, VCN measurement allows a reliable quantification of transduced cell engraftment in tissues, including the brain. Here below, for Reviewers' consultation, we included an example of correlation between transduced cell engraftment in the brain measured as VCN values and %GFP+ cells at flow cytometry obtained on an unrelated experimental cohort that received HSPCs transduced with a LV encoding for a fluorescent transgene and showing a similar VCN in the infused product. These data allow us to estimate that the transduced cell engraftment in the brain of the treated 5xFAD mice is comprised between 30 and 80%, upon correction for the input cell VCN.



In addition, to support the engraftment data by a different technique, we evaluated the expression of hTREM2 on the brain tissue extract of ICV transplanted animals (N=6) by quantitative RT-PCR (RT-qPCR) using a SYBR Green Kit with primers and probe from Thermofisher (Catalog number: 4448892, Hs01003898_m1, Product size: 91 bp, Dye: FAM). For this gene expression analysis, we preliminary validated the hTREM2 primers on human THP1 cells, a monocytic cell line that constitutively expresses hTREM2, and on BV2 murine cells transduced with the LV_hTREM2, both used as positive controls for hTREM2 expression. All values were normalized to the housekeeping gene GAPDH. hTREM2 expression was then measured on the brain of the study animals transplanted ICV with HSCs transduced with the therapeutic LV-hTREM2, collected at sacrifice 10 months after the transplant. Importantly, a significant correlation was observed between hTREM2 expression measured by RT-qPCR and the LV-hTREM2 content, measured as vector copy number per cell on the brain tissue of the transplanted animals (R2 = 0.99) (see **New Figure 5H**).

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Response: We agree with the Reviewer on the relevance of separating as much as possible contribution of the blood/BM-derived cells from that of the cells delivered in the CNS. For this reason, we:

- extensively perfused the animals at sacrifice and we included in the revised version of the manuscript more details on animal perfusion and tissue processing to clarify this critical aspect; mice were euthanized under deep anesthesia by extensive intracardiac perfusion with cold Phosphate Buffered Saline (20-30ml PBS for at least 10-15 minutes/mouse), after clumping the femur to preserve bone marrow cells. The procedure is usually stopped only when complete liver blanching is observed; the brain and the spinal cord appear clear after perfusion. We did not use formalin since the same animals were used for IF and flow cytometry analysis, both performed on fresh tissues. The addition of heparin to help inhibit blood clot formation is optional for this procedure and we usually do not add heparin to the washout solution, but we could take advantage of this suggestion and include the use of heparin in our standard operative procedure for future experiments;
- in flow cytometry analyses of both brain and spinal cord we used a gating strategy that excludes leukocytes and circulating cells;

Importantly, the animals analyzed in this study were transplanted with the transduced HSPCs by ICV administration, a procedure that does not result in hematopoietic engraftment of the transplanted cells, thus residual circulating blood cells that would have remained after extensive perfusion and gating would be in any case expressing the TREM2 vector and not originated from the transduced and transplanted HSPCs; this is confirmed by the VCN retrieved in the BM, which is virtually negative in all study animals.

5. The authors use a lentivirus to overexpress human TREM2 in 5x FAD mice. Given the poor amino acid sequence similarity between mouse and human TREM2, the authors need to evaluate whether immunogenicity from the transgene expression contributes to the phenotype in the transgenic mice.

Response: We used a lentivirus to overexpress human TREM2 in order to facilitate the clinical translation of our approach. Although human and mouse genes share ~77% sequence identity, the human protein is equally functional and its increase augmented the phagocytic activity of murine microglia in functional in vitro assay as showed by us and other groups (Lee et al 2018, Wang et al 2020). Furthermore, a detailed analysis of the phenotypic impact of increased human TREM2 gene dosage in animals is well documented in literature and supported by in vivo study showing that 5XFAD mice develop less Aβ pathology when crossed to transgenic mice overexpressing human TREM2 (Lee et al 2018). In our study, we did not observe any clinical signs or reported intercurrent death that could be specifically related to immunotoxicity caused by transgene expression, nor clearance of the transduced cells from the treated mice tissues as a consequence of an active immune response. We may also speculate that the transplant procedure per se, associated to myeloablation, could have contributed to the induction of tolerance to the human protein.

6. The manuscript does not report an un-transplanted 5xFAD mouse group as a control for pathobiology and behavioral changes in the authors' experimental conditions. Historical references to the phenotypes are insufficient to help the reader to understand if conditioning, transplantation and TREM2 overexpression improves the behavior and/or the pathobiology of the 5xFAD mouse. This is a significant limitation of the study that needs to be addressed by the authors.

Response: The transplant procedure and animal manipulation per se may have an effect in modifying the behavior of the treated and control animals, particularly those tests involving spatial and working memory. For this reason, in studies involving such an extensive manipulation it is important to account for the manipulation itself and treatment administration as relevant factors to be accounted for properly estimating the treatment effects over controls. To this aim, we used as disease reference/study controls mock transplanted animals instead of un-transplanted 5XFAD mice, which received the same treatment based on 4 doses of Busulfan (25mg/kg) and ICV transplantation of transduced HSPCs, with the exception of the use of a reporter vector rather than the therapeutic hTREM2 LV for transduction, to properly account for any confounding effect of the manipulation on the disease manifestations. In line with these criteria, we selected the most informative behavioral tests to be used to evaluate treatment effects by comparing mock-controls 5XFAD mice with mock-treated wild type animals. Importantly, the data obtained in these cohorts were interpreted in light of relevant literature.

7. The authors declare in the reporting summary that they articulate the exact sizes of each experimental group. However, the manuscript and supplemental material does not clarify any group sizes. The authors need to help the reader to understand the strength of any statistical associations reported by explicitly stating the group sizes for each statistical analysis presented.

Response: We thank the Reviewer for his/her precious suggestion. We included this information in the manuscript.

8. The authors mention that mice were dosed but did not show engraftment were excluded from the experimental groups. Can the authors state the total numbers of mice that were dosed and the total numbers of mice that demonstrated engraftment so that the reader can understand the reproducibility of the therapeutic approach?

Response: We did not exclude any animals from AD experiments, so the therapeutic effect cohort is untouched. The data reported in Figure 5 are representative of N>10 mice per group. The information reported in the filled form refers to combinatorial transplantation experiments in wild type mice in which few non- engrafted animals (N<4 mice, defined as having <20% donor engraftment) were excluded since the absence/low engraftment in the hematopoietic organs of myeloablated animals is unexpected and is likely due to technical issues related to Busulfan administration and/or cell injection.

Minor points

1. The authors rather surprisingly use dollar signs to represent statistical significance, which seems inappropriate. Can the authors use a different character to represent significant associations while avoiding the graphical representation linking money to statistical significance?

Response: We are sorry for this unintentional use. We changed the character used to represent statistical significance.

2. Can the authors provide the pre-transplantation vector copy numbers of the cells administered to each experimental group?

Response: For the 5XFAD experiments, we performed VCN analysis on the pre-infused cell product and we included this information in the results section. For the combinatorial transplant experiments, we do not have VCN data since we based our analysis on fluorescence values obtained by flow cytometry.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this revision, Milazzo et al. have provided some new data in response to previous reviewers' comments. However, even with revision, the manuscript is not quite satisfying. Unfortunately, many of the previous reviewers' questions were in fact circumvented and heavily relied on assumptions derived from previous literature. I will list my concerns as follows:
About the transplantation:
1. It is interesting to compare different injection sites for microglial replacement after busulfan treatment with dual injections. Recent studies on the CNS transplantation have done a lot of great work, especially in characterizations of transplanted cells (some of them were iPSC-derived myeloid cells) vs endogenous microglia, which the authors might want to refer to. Please include it in the discussion.
2. Regarding the focus on the routes for HSPCs to reach the CNS, which previous reviewers also commented on, the assessment was roughly superficial. I think the previous reviewers' comment was valid. They were expecting results about migrating patterns through certain routes with different injections, which means the authors need a greater number of the mice and more time points.
3. The microglia-like gene profile was inferred merely from morphology and qPCR from less than 10 genes. The IHC verification was still limited to RNAscope instead of antibody staining. Previous reviewers have suggested bulk RNAseq to obtain more info for the transplanted HSPC and how they behave, particularly in this study. A selected microglia signature would not be helpful because many genes beyond the selected ones could contribute to microglial response towards amyloid pathology. For example, the authors misunderstood the purpose for asking p2ry12 vs p2ry13 by the previous reviewer 1, as p2ry12 has been shown to be an important homeostatic marker for assessing microglial response to amyloid pathology.
About benefiting AD:
4. The benefit for AD pathology by intra-CNS transplantation of engineered HPSC was poorly characterized. The model was simply a hTREM2-overexpressed HPSC injection into the 5xFAD model even without excluding endogenous murine TREM2 expression. Many of the major points have not been addressed:
a. Given that different brain regions would have different engraftment efficiency according to fig.2, shouldn't the authors assess the whole brain as well as specific regions to correlate to their transplantations?
b. Besides DAB staining, there is no biochemical assessment of amyloid pathology in their study.
c. Since it is hTREM2 engineered HSPC, would the engraftment remain the same as previously shown with WT HSPC? In addition, since it is a 5xFAD model, how much do those HSPC migrate to the amyloid plaques? The authors never address those points. The authors could distinguish exogenous HSPC-derived microglia-like cells vs endogenous repopulated microglia by hTREM2 vs mTREM2 as suggested by previous reviewer 2. More importantly, flow cytometry would not help to distinguish pvMac vs parenchymal microglia. The authors should perform IHC experiments. I do not

understand how the authors got r=0.9049 with that curve with their SYBR Green Kit, which the dots were obviously exponential instead of linear.
d. There is no evidence to suggest that the reduction of total amyloid burden is a direct cellular effect from hTREM2 HPSC transplanted. The authors did not provide any assessment on those engrafted cells and how they interact with amyloid plaques, etc.
5. There are random citations, which are not very appropriate. For example, Ref #27 was a review discussing LSD. No text was related to "the engineered microglia-like cells progressively appearing in the CNS of transplant recipients" except one brief sentence in the abstract. Only Ref#29 was a support to that conclusion. In their response, Zhang et al., 2014 was another wrong citation. They did not directly compare the expression level between p2ry12 and p2ry13.
Minor:
1. Typos: in the method section of immunohistochemistry, 4% PAF should be 4% PFA.

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NCOMMS-392072

Intra-CNS transplantation of engineered HSPCs uniquely drives their fate and benefits Alzheimer's disease in mice.

Milazzo et al.

In this new version of the manuscript, the authors discuss the rationale for the 8 major points raised by the previous review and satisfactorily respond to Questions 7 and 8. However, questions 1-6 are reasonable and require new data/analyses but the authors are unable to provide satisfactory answers or update the manuscript to address these concerns, especially related to the engraftment observed in the genetic model of amyloidopathy and linkage between conditioning, epilepsy and engraftment. Without such data, the significant weaknesses of the manuscript remain unresolved.

POINT-BY-POINT REPLY to the REVIEWERS' COMMENTS

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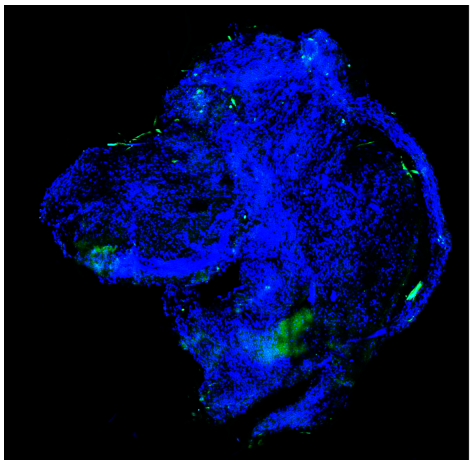
Response: We thank the Reviewer for this observation. We included this recently published information in the discussion session (rows 391-395; 512-523).

2. Regarding the focus on the routes for HSPCs to reach the CNS, which previous reviewers also commented on, the assessment was roughly superficial. I think the previous reviewers' comment was valid. They were expecting results about migrating patterns through certain routes with different injections, which means the authors need a greater number of the mice and more time points.

Response: To address this Reviewer's comment, we performed a short-term evaluation of cell engraftment in the hematopoietic and CNS compartments of busulfan-treated wild type mice that had received murine HSPCs labeled with GFP LVs via IV, ICV or ITL delivery. We first identified by flow cytometry (FC) the earliest time point at which donor-cell chimerism was present (and quantifiable) in the CNS and then selected this time point for subsequent IF-based imaging of the GFP^+ cells. The study evaluated the transplanted animals at +1, 5, 14 days post-transplant (**new Figure 2A-C**). At the earliest time points, besides the bone marrow in IV-transplanted animals, the donor cells were clearly measurable only in the brain of ICV transplanted mice, while by day 14 discrete levels of CNS engraftment were observed in all the tested conditions. Based on these data, day 14 was chosen as the earliest timepoint to comparatively study, by IF analysis, the biodistribution of the CNS engrafted cells in the mice transplanted with the different routes. We also analyzed animals at +30 days post-transplant to follow the distribution across regions over time. In order to efficiently detect the distribution pattern of short-term immigrant cells in the CNS of transplanted mice, we decided to analyze a whole-mount 2mm-thick sagittal section of the brain, instead of performing standard cryo-sectioning to avoid the risk of missing tiny cell populations. To do this, we optimized a recently described tissue clearing technique (Peviani, Spano et al. 2020) allowing for high-resolution fluorescent microscope imaging of brain slices. while preserving the original dimensions of the tissue. Interestingly, depending on the route, we observed a differential distribution of the GFP^+ cells in the analyzed tissues. The following observations are thus now available (and have been included in the manuscript in rows 142-219 of the results section, in **new Figure 2**, and commented in the discussion, at rows 434-483; details of the tissue clearing procedure were also included in the methods section):

- **ICV**
  - Day 14: the transplanted cell progeny was mostly detectable in the close proximity of the ventricular structure, in the septum and projecting rostrally and towards the basal forebrain (**new Figure 2B**). A few clustered cells could also be observed in the midbrain and pons. Although the time point of observation was very precoucious, already discrete branchings of the GFP^+ cells were visible suggesting an actively occurring differentiation process (**new Figure 2G**);
  - Day 30: the GFP^+ cells preferentially distributed across brain regions through the cerebral ventricular system and exploiting a trajectory which may suggest proximity to the rostral migratory stream. Clustered cells were also observed in the proximity of the third and fourth ventricles, in the diencephalon and in the hindbrain, from which they distributed into nearby regions, colonizing the striatum, the anterior olfactory area and the brainstem (**new Figure 2C, G**).
  - Day 45 (already present in the previous version of the manuscript): the GFP^+ cells continue spreading rostrally, in a trajectory in the proximity of the rostral migratory stream, with high frequency particularly in the forebrain and olfactory region. Notably GFP^+ cells start to expand in regions that were poorly colonized at previous time points, as the cortex and the cerebellum; these cells occupy different locations than the $Cherry^+$ progeny of the IV injected counterpart (Figure 2D and Figure S2A);
  - Day 90 (already present in the previous version of the manuscript): the GFP^+ cells now occupy a significantly larger area in the parenchima, with still a preference in colonizing the cortex (as at +45 days), the hippocampus, the corpus callosum, and the olfactory bulbs. Notably, they continue to maintain a mutual exclusive distribution with IV co-injected cells, which instead, preferentially expand in the pons, medulla and cerebellum (Figure 2E and Figure S2A).
- **ITL**
  - Day 14: in the brain only a very limited donor cell quantity was documented by FC and, similarly, a very few and sparse GFP^+ cells were identified by IF, without any obvious clustering. Also in the spinal cord, we failed to identify a pattern, as the few detected GFP^+ cells were found in different locations with no obvious clustering (a representative IF picture is shown here below for the Reviewer, but we would not include this data in the manuscript unless he/she deems otherwise).



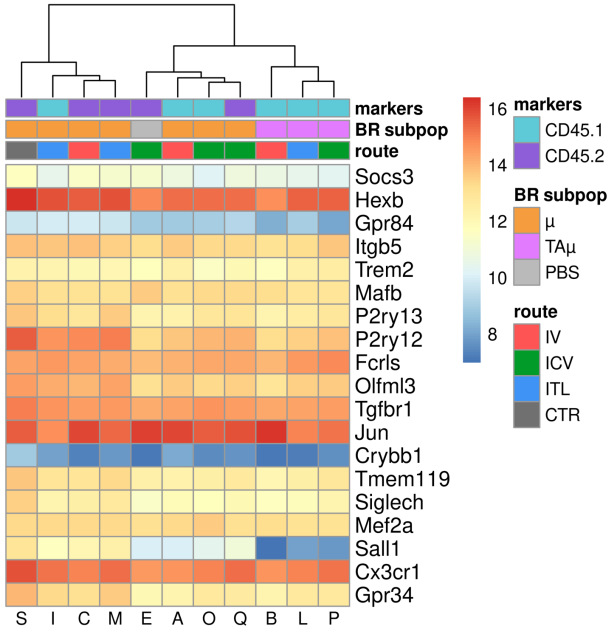
Day 15 spinal cord IHC picture from an ITL transplant recipient (DAPI in blue, GFP in green)

- Day 30: the injected cells were mostly localized in the olfactory bulb and forebrain regions (**new Figure 2C, G**). A few clusters of cells could also be observed in the brainstem, which is consistent with its proximity to the spinal cord. Unlike the ICV route, no cells were retrieved in the subventricular zone and lateral ventricle regions.
- Day 45 (already present in the previous version of the manuscript): the GFP^+ cells continue to spread into nearby regions, occupying the striatum and the hypothalamus, with many cells localized particularly in the forebrain and olfactory region. As for the ICV+IV setting, ITL derived cells occupy different locations than the $Cherry^+$ progeny of the IV injected counterpart, that instead remain mostly confined to the hippocampus (Figure 2D and Figure S2A);
- Day 90 (already present in the previous version of the manuscript): the GFP^+ cells now are present at high frequency in almost all regions, with few exception as the cortex and in part the midbrain.(Figure 2E and Figure S2A).

- **IV**
  - Day 14: very few donor/GFP^+ cells were detected by FC (new Figure 2A) and IF (not shown) in the brain and spinal cord of transplant recipients. These few cells showed no specific clustering and were mostly localized in the proximity of meninges and blood vessels, suggesting a migration pattern possibly involving these structures (please refer to discussion). Unlike what was observed in ICV-transplanted mice, GFP^+ cells here displayed a round shape and no branchings, a phenotype likely consistent with immature cells (**new Figure 2G**).
  - Day 30: at this time point almost all the GFP^+ cells were localized in the hyppocampus, suggesting a specific homing of the IV-transplanted cells in this region (new Figure 2C, G). A few cells were also detected in the forebrain region and in the brainstem likely infiltrating throughout the brain parenchyma from the meninges and blood vessesl (**new Figure 2C**).
  - Day 45 (already present in the previous version of the manuscript): the GFP^+ cells expand from the hippocampus area in a trajectory that follow the rostral migratory stream reaching the forebrain and olfactory bulb region. A cluster of cells is also present in the midbrain region close to the cerebellum. The cortex, the pons and the brainstem are poorly colonized (Figure 2D)
  - Day 90 (already present in the previous version of the manuscript): IV-injected cells increase in their frequency and now occupy the regions that were poorly colonized in previous time points as the brainstem and in particular in the cortex (Figure 2E and Figure S2B).

3. The microglia-like gene profile was inferred merely from morphology and qPCR from less than 10 genes. The IHC verification was still limited to RNAscope instead of antibody staining. Previous reviewers have suggested bulk RNAseq to obtain more info for the transplanted HSPC and how they behave, particularly in this study. A selected microglia signature would not be helpful because many genes beyond the selected ones could contribute to microglial response towards amyloid pathology. For example, the authors misunderstood the purpose for asking p2ry12 vs p2ry13 by the previous reviewer 1, as p2ry12 has been shown to be an important homeostatic marker for assessing microglial response to amyloid pathology.

Response: To address the Reviewers' concerns, we performed a genome-wide expression analysis by means of an Illumina RNA sequencing (RNA-seq) platform on a pool of sorted μ and TAμ populations from IV, ITL and ICV transplanted mice sacrificed at 45 days post-transplant (busulfan conditioned CD45.2 recipients were transplanted with CD45.1 GFP labeled HSPCs). Donor GFP^+ cells (μ and TAμ) were sorted according to the expression of the CD45.1 and CD11b antigens. RNA extraction and sequencing were performed by IGA Technology Services. The transcriptional profile of the donor-derived cells was compared with the profile of microglia from adult naïve mice, sacrificed together with the transplanted ones as internal control (N=2-3 animals/group). For consistency, we focused our analysis on microglia signature enriched genes and transcription factors expressed by microglia. Nicely, we confirmed our previously reported findings, as we observed a similar expression pattern between control microglia and the cells sorted from the transplanted animals. Moreover, regardless of the route of HSPC delivery, TAμ populations from the transplanted mice clustered together and separately from the μ cells, again confirming the hypothesis that these two subsets may represent different maturation stages of transplant-derived cells. A lower expression of the transcription factor Sall1, commonly associated to yolk sac-derived microglia, was noticed in transplant-derived cells compared to controls, as expected due to the different ontogeny and as also described by other groups for similar populations(Cronk, Filiano et al. 2018). Interestingly, the expression of Sall1 was lower in TAμ versus μ cells, further supporting our hypothesis. We also included in the analysis the P2RY12 gene, as suggested by the Reviewer, and observed its expression at variable levels across samples.
Overall, these independently generated RNAseq data, reported in the heatmap below, are consistent with our previous findings, but since they may not add substantial new information we would refrain from including them in the final manuscript, unless the Reviewer suggests otherwise.



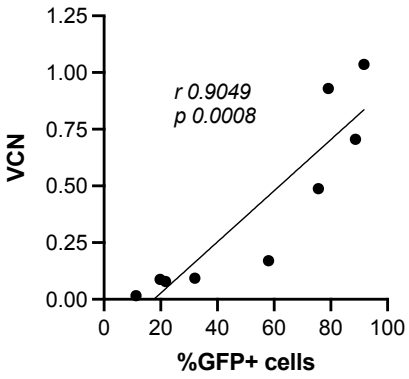
Heatmap of normalized expression values for microglia-specific genes. The heatmap illustrates the normalized expression values for a curated list of genes specific to microglia, including microglia marker genes and microglia-specific transcription factors, as reported in Lund et al. 2018.

About benefiting AD:
4. The benefit for AD pathology by intra-CNS transplantation of engineered HPSC was poorly characterized. The model was simply a hTREM2-overexpressed HPSC injection into the 5xFAD

model even without excluding endogenous murine TREM2 expression. Many of the major points have not been addressed:
a. Given that different brain regions would have different engraftment efficiency according to fig.2, shouldn't the authors assess the whole brain as well as specific regions to correlate to their transplantations?
b. Besides DAB staining, there is no biochemical assessment of amyloid pathology in their study.
c. Since it is hTREM2 engineered HSPC, would the engraftment remain the same as previously shown with WT HSPC? In addition, since it is a 5xFAD model, how much do those HSPC migrate to the amyloid plaques? The authors never address those points. The authors could distinguish exogenous HSPC-derived microglia-like cells vs endogenous repopulated microglia by hTREM2 vs mTREM2 as suggested by previous reviewer 2. More importantly, flow cytometry would not help to distinguish pvMac vs parenchymal microglia. The authors should perform IHC experiments. I do not understand how the authors got r=0.9049 with that curve with their SYBR Green Kit, which the dots were obviously exponential instead of linear.
d. There is no evidence to suggest that the reduction of total amyloid burden is a direct cellular effect from hTREM2 HPSC transplanted. The authors did not provide any assessment on those engrafted cells and how they interact with amyloid plaques, etc.

Response: As per Reviewer's suggestion, we included additional data to further characterize the benefit associated to intra-CNS administration of TREM2-overexpressing HSPCs in the 5xFAD model.
Firstly, to account for any effect related to the cell distribution, we included representative images of treated vs untreated 5xFAD mice showing the distribution of β amyloid deposits and GFAP staining in whole brain parasagittal sections. These images confirm a substantial reduction of Aβ in different brain regions of the transplanted mice, consistently with the expected transduced cell distribution that in the long term post-transplant is diffused and wide in the whole brain. Similarly, a reduction of astrogliosis was observed throughout the examined sections, consistent with the data quantified in individual images. We included these data in the result session-at rows 352-361 and in **new Figure S3D**.
Secondly, we performed in situ hybridization (ISH) using a probe directed against the Woodchuck Hepatitis Virus (WHV) post-transcriptional regulatory element (WPRE) as a strategy to specifically identify the LV-transduced, TREM2 expressing cells in brain sections of representative 5xFAD treated animals. This strategy was adopted to overcome the lack of specific antibodies faithfully discriminating human versus murine TREM2. Notably, since the WPRE sequence is not present in the mouse genome, ISH for WPRE allows to detect specifically the distribution of LV-transduced cells in the tissue (Tiesjema, la Fleur et al. 2007); moreover, as the WPRE sequence was added after the coding sequence of TREM2 in the LV expression cassette to stabilize the therapeutic transcript, the WPRE signal at in-situ corresponds to TREM2 expression. The ISH was combined with IF staining with an anti-Aβ antibody. Through this approach, we could detect abundant TREM2-expressing cells in the brain of the treated 5xFAD mice; these cells, which were distributed thought the brain parenchyma, were particularly abundant in the cortex and hippocampal regions, which are the brain areas where Aβ plaques were mostly detected (**new Figure 5N**). Interestingly, the TREM2 expressing cells were detected in the close proximity of the Aβ signal (**new Figure 5O**). These data, support the hypothesized mechanism of action of TREM2 HSC gene therapy, namely that the TREM2 overexpressing cells interact with amyloid plaques in the CNS of transplanted mice and have the potential to reduce amyloid deposition. This is in line with what reported in literature upon transplantation of WT HSPCs (Yoo, Neumayer et al. 2023) in the same animal model. These data

were included in rows 362-374 of the results section and in the new Figure 5N,O and S3E and commented in the discussion, at rows 537-545. Details of ISH for WPRE were also included in the methods section.
To address the point raised by the Reviewer on the SYBR Green data, in the previous revision, we included here an example of correlation between the engraftment of transduced cells measured by ddPCR, expressed as VCN, and FC, expressed as frequency of GFP^+ cells, obtained on an unrelated experimental cohort of animals.



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Response: We kept ref#29 as suggested by the Reviewer and removed ref#27 and Zhang et al. 2014.

Minor:
1. Typos: in the method section of immunohistochemistry, 4% PAF should be 4% PFA.

Response: We modified the text accordingly in the method section.

Reviewer #2 (Remarks to the Author):

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Intra-CNS transplantation of engineered HSPCs uniquely drives their fate and benefits Alzheimer's disease in mice.

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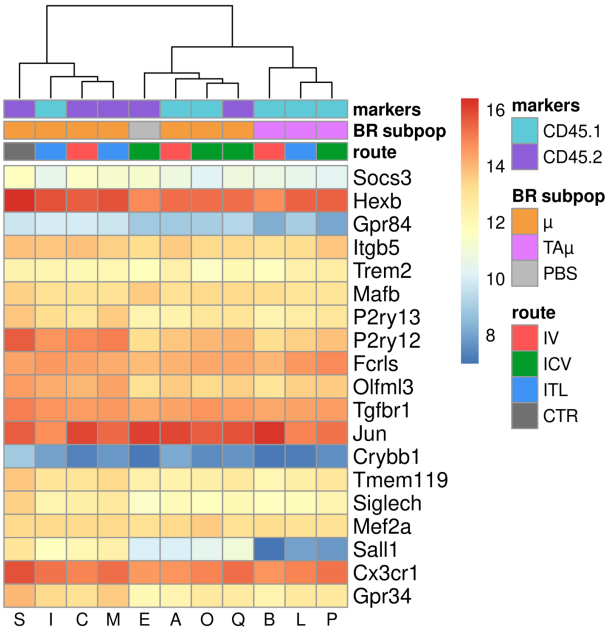
Response: We thank the Reviewer for appreciating our efforts and allowing us to further improve it. In particular, we are re-addressing the questions #1 - 6 raised in the previous revision from this Reviewer and for which our previous answers were not satisfactory, as follows:

1) We performed a genome-wide expression analysis by means of an Illumina RNA sequencing (RNA-seq) platform on: a) a pool of sorted μ and TAμ populations from IV, ITL and ICV transplanted mice sacrificed at 45 days post-transplant (busulfan conditioned CD45.2 recipients were transplanted with CD45.1 GFP labeled HSPCs; donor GFP+ cells (μ / TAμ), sorted according to the expression of the CD45.1 and CD11b antigens); b) microglia from adult naïve mice, sacrificed together with the transplanted ones as internal controls; c) microglia from adult mice receiving PBS via ICV injection as additional sham-controls. RNA extraction and sequencing were performed by IGA Technology Services. For consistency, we focused our analysis on microglia signature enriched genes and transcription factors expressed by microglia. Nicely, we confirmed our previously reported findings, as we observed a similar expression pattern between microglia from PBS injected and naïve untreated mice, and the cells sorted from the transplanted animals. These independently generated RNAseq data, reported in the heatmap below, are consistent with our previous findings and exclude that the procedure per se could significantly affect the gene expression profile of resident microglia. We would refrain from including these data in the final manuscript, unless the Reviewer suggests otherwise.

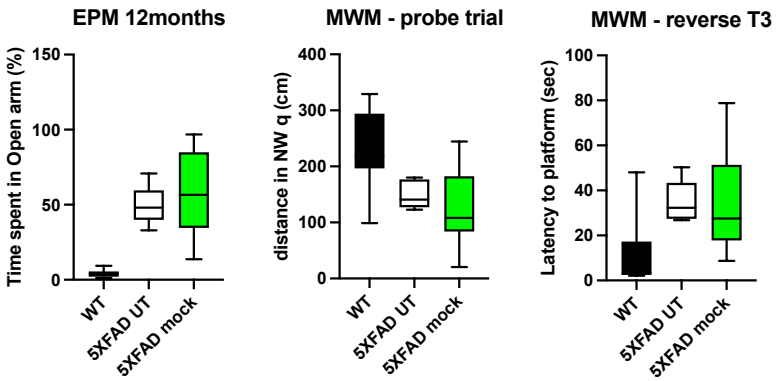


Heatmap of normalized expression values for microglia-specific genes. The heatmap illustrates the normalized expression values for a curated list of genes specific to microglia, including microglia marker genes and microglia-specific transcription factors, as reported in Lund et al. 2018.

2) We extensively revised recent literature and would respectfully underline firstly that, despite the administration of anticonvulsant prophylaxis has become common during high-dose busulfan-based conditioning regimes in patients (and mice) undergoing hematopoietic cell transplant, no specific pathological findings have been reported as associated to these epileptic events that are anyhow confined to a very specific, transient and concurrent-with-treatment administration time window. No link between this well-known side effect of busulfan and neural damage and/or the process of donor cell engraftment has ever been established. In particular, donor cell engraftment in the CNS occurs at timings (which are well depicted in Figure 1 and in the new Figure 2) that are much delayed as compared to the neurotoxic effects of busulfan, if any, which are eventually confined to the short-term post-administration. Based on these considerations, we thus believe that identifying possible signs of busulfan-related neuronal damage at the time of treatment administration would go beyond the scope of this manuscript, which focuses its attention at a time of analysis that goes long after the time when these events could have occurred. As commented earlier, in the attempt to prevent/limit the occurrence of seizures during busulfan administration, we administered anticonvulsants to 5xFAD mice with daily administration of class IV pharmaceutical grade diazepam solution (available at 5 mg/mL) in the drinking water starting for a total of 10 days with progressive decreases in drug dose (0.25 mg/kg used for the first 8 days, then dose decreased in the two following days to 0.125mg/kg - 0.063 mg/kg and removed).
3) As specific antibodies effectively recognizing human TREM2 from the murine counterpart were not available to us, we could not apply neither flow cytometry nor detailed immunohistology mapping of the transplanted cell progeny in the treated animals. However, we could image the TREM2 expressing cells on brain slices from the treated mice by in situ hybridization (ISH) using a probe directed against the Woodchuck Hepatitis Virus (WHV) post-transcriptional regulatory element (WPRE) as a strategy to specifically identify the LV-transduced, TREM2 expressing cells in brain sections of representative 5xFAD treated animals. Notably, since the WPRE sequence is not present in the mouse genome, ISH for WPRE allows to detect specifically the distribution of LV-transduced cells in the tissue (Tiesjema, la Fleur et al. 2007); moreover, as the WPRE sequence was added after the coding sequence of TREM2 in the LV expression cassette to stabilize the therapeutic transcript, the WPRE signal at in-situ corresponds to TREM2 expression. The ISH was combined with immunofluorescence staining with an anti-A□ antibody. Through this approach, we could detect abundant TREM2-expressing cells in the brain of the treated 5xFAD mice; these cells, which were distributed thought the brain parenchyma, were particularly abundant in the cortex and hippocampal regions, which are the brain areas where A□ plaques were mostly detected (new Figure 5N). Interestingly, the TREM2 expressing cells were detected in the close proximity of the A□ signal (New Figure 5O). These data were included at rows 362-374 of the results section of the manuscript and in the new Figure 5N, O and S3E and commented in the discussion, at rows 537-545.
4) As described in the previous point-by-point reply, the animals on which we reported in the manuscript had been extensively perfused with saline, or saline plus heparin (in the short-term kinetics) at sacrifice.
5) As discussed in the previous point-by-point reply, our and others' experimental data in preclinical models and patients have proven the lack of immune responses in a myeloablative HSPC transplant setting, also when routes other than the standard are employed. The durability of the vector-dependent signals (VCN, WPRE at in situ, TREM2 mRNA expression etc.) and of the therapeutic effects up to 12 months post-transplant support this concept. Therefore, in the absence of evidences suggesting a direct effect on the transplanted cells/their

progeny, we consider that searching for a TREM2 specific immune response would go beyond the scope of the manuscript.

6) As previously discussed, and as usually performed in studies like the one we performed on 5xFAD mice, the proper disease control that has to be employed to account for treatment-related biases is represented by mock transplanted animals. To confirm that busulfan administration and ICV cell injection per se did not affect/mitigate the AD phenotype we here provide data that were originally generated on separate cohorts of 5xFAD UT and mock/GFP transplanted 5xFAD mice to characterize the colony to be subjected to treatment. As the Reviewer could appreciate, the behavioral phenotype evaluated with the same tests described in the manuscript is superimposable in untreated and mock/GFP 5xFAD controls. The data are below, and as per Reviewer's suggestions could (or not) be included in the manuscript.



Results of the Elevated Plus Maze (EPM) and Morris Water Maze (MWM – probe trials results and reverse platform test results, trial #3) trials in wild type (WT) and 5XFAD mice (untreated – UT or mock/GFP transplanted, as indicated) at 12 months post mock-transplant/14 months of age.

References for this document:

Cronk, J. C., A. J. Filiano, A. Louveau, I. Marin, R. Marsh, E. Ji, D. H. Goldman, I. Smirnov, N. Geraci, S. Acton, C. C. Overall and J. Kipnis (2018). "Peripherally derived macrophages can engraft the brain independent of irradiation and maintain an identity distinct from microglia." J Exp Med **215**(6): 1627-1647.

Peviani, M., G. Spano, A. Pagani, G. Brugnara, C. Covino, R. Galli, A. Biffi and L. S. Politi (2020). "Lipophilic dye-compatible brain clearing technique allowing correlative magnetic resonance/high-resolution fluorescence imaging in rat models of glioblastoma." Sci Rep **10**(1): 17974.

Tiesjema, B., S. E. la Fleur, M. C. Luijendijk, M. A. Brans, E. J. Lin, M. J. During and R. A. Adan (2007). "Viral mediated neuropeptide Y expression in the rat paraventricular nucleus results in obesity." Obesity (Silver Spring) **15**(10): 2424-2435.

Yoo, Y., G. Neumayer, Y. Shibuya, M. M. Mader and M. Wernig (2023). "A cell therapy approach to restore microglial Trem2 function in a mouse model of Alzheimer's disease." Cell Stem Cell **30**(8): 1043-1053 e1046.

REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author):

NCOMMS-392072

Intra-CNS transplantation of engineered HSPCs uniquely drives their fate and benefits Alzheimer's disease in mice.

Milazzo et al.

The revised manuscript has significantly improved. The reader will appreciate the inclusion of the grafted and host microglial transcriptomes into the manuscript. However, the authors' response to Question 2 remains somewhat enigmatic for the reader.

The authors take the position that busulfan does not damage the vulnerable neural circuits of Alzheimer's disease. Given the scientific significance of this statement for the manuscript and the field, the reader would benefit from understanding this point clearly and directly from the authors in the manuscript's discussion.

As a minor point, the abstract overstates that the data represents "the first example of efficacy of HSPC gene therapy in an adult multifactorial neurodegenerative disease" when the heavy genetic load of the mouse model is, in fact, very similar to a model of rare genetic disease. The reader would benefit from a more tempered, realistic representation of the findings in the abstract.

Reviewer #3 (Remarks to the Author):

The authors have performed additional analyses to attempt to address reviewer's previous questions. In particular they have performed a more extensive characterization of the distribution of immune cells throughout the brain given different routes of administration which for this reviewer is sufficient. However, the limited analysis of RNAseq data to a handful of genes from a small number of mice per condition is not adequate to address the reviewer's questions surrounding a microglia-like profile. Many of these genes could also be present in macrophages for example. A more complete comparison would be to directly examine the expression profiles of microglia, macrophages, and transplanted cells.
In regard to the 5xFAD data, it is unclear why mock and GFP conditions were combined. These should not be plotted together. Additional support for the role of plaque-associated cells could be added by the use of activation-associated markers, such as CD68 or CLEC7A. It is also worth noting that the 5xFAD model is a model of amyloid pathology, not of Alzheimer's disease as it does not develop tau pathology or marked neurodegeneration. Furthermore it is a model that expresses

two transgenes with 5 mutations, not 5 transgenes as stated in line 311.

POINT-BY-POINT REPLY TO THE REVIEWERS' COMMENTS

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R: We are grateful to the Reviewer for this observation that we agree covers a very relevant aspect, particularly as far as clinical translation of our findings is concerned. Firstly, we would like to underline the proof-of-concept nature of our findings, which will be properly adapted in a clinical development plan, including in terms of modulating the intensity and nature of the conditioning regime. Secondly, we would comment that:

1) *the overall treatment resulted in substantial disease-modifying effects: a significant amelioration of the behavioral and histopathological disease manifestations was observed, with improved performance of the treated versus control mice at the Elevated plus maze and Morris Water Maze tests, as well as reduction of neuroinflammation (astrogliosis and microgliosis);*
2) *despite the administration of anticonvulsant prophylaxis to 5xFAD mice that was implemented to preclude or minimize any potential short-term neurotoxic effects of busulfan, none of these potential side effects appeared in the treated mice and busulfan-receiving mock controls, at least phenotypically as per regular daily observation.*
3) *as far as long-term neurotoxic effects of busulfan are concerned, behavioral studies and regular clinical observation did not reveal busulfan-associated deficits of new onset in the treated/busulfan-receiving controls versus untreated 5XFAD or wild type animals.*
4) *no obvious nor severe signs of neural damage were observed at histopathological observation of the treated and control mice.*

Although specific neurotoxic effects of busulfan across epileptogenic and/or other neural circuits in this Alzheimer's disease models remain undefined at the microscopic evaluation, such effects have not overtly compromised treatment outcomes nor treated and control mice overall performances.
These observations we included in the discussion section (rows 581-588).

As a minor point, the abstract overstates that the data represents "the first example of efficacy of HSPC gene therapy in an adult multifactorial neurodegenerative disease" when the heavy genetic load of the mouse model is, in fact, very similar to a model of rare genetic disease. The reader would benefit from a more tempered, realistic representation of the findings in the abstract.

R: We thank the Reviewer for this observation. We revised the abstract to more accurately represent our study as an opportunity to treat complex neurodegenerative diseases by means of an innovative HSC gene therapy approach. Of note, the 5XFAD model is considered a relevant and reliable AD animal model that well conveys, because of it multigenic nature, a complex neurodegenerative condition, whereas previous applications of

HSC gene therapy were confined to monogenic neurodegenerative/demyelinating diseases. The wording of the abstract was modified to conveying this concept more properly.

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The authors have performed additional analyses to attempt to address reviewer's previous questions. In particular they have performed a more extensive characterization of the distribution of immune cells throughout the brain given different routes of administration which for this reviewer is sufficient. However, the limited analysis of RNAseq data to a handful of genes from a small number of mice per condition is not adequate to address the reviewer's questions surrounding a microglia-like profile. Many of these genes could also be present in macrophages for example. A more complete comparison would be to directly examine the expression profiles of microglia, macrophages, and transplanted cells.

R: The characterization of donor-derived microglia-like populations was the focus of a previous work, where we deeply analyzed the transcriptomic features of these cells and compared them with control microglia across development stages (Capotondo, Milazzo et al. 2017). This comparison included also macrophage populations, identifying different patterns of gene expression able to clearly discriminate donor-derived microglia-like cells' gene expression profile from macrophage's one. The current work aimed at confirming these findings and comparing the maturation of HSPC progeny cells obtained upon cell transplantation via different delivery routes. For this reason, we specifically focused on short list of genes analyzed on a pool of sorted µ and TAµ populations from IV, ITL and ICV transplanted mice versus microglia from control adult mice. To provide a deeper overview of the differences that could be observed between host and newly formed microglia-like cells in the different settings, as suggested by the Reviewer, we further extended our analysis to a published gene set used in several other works to describe microglia and engrafting myeloid cells across several models of microglia depletion (Bruttger, Karram et al. 2015, Bennett, Bennett et al. 2018, Cronk, Filiano et al. 2018, Lund, Pieber et al. 2018). For each transplant setting, we compared the transcriptome profile of host mature microglia with newly formed microglia subpopulations (µ and TAµ). As shown in the heatmap in the new Supplementary Figure 3 and reported in the results session (rows 287-307), we observed a very similar expression pattern between resident microglia and corresponding donor derived µ and TAµ myeloid subpopulations retrieved from each of transplant settings. Of note, as reported in previous analysis, we confirmed a lower expression of the transcription factor Sall1, commonly associated to yolk sac-derived microglia, in TAµ versus µ cells, again supporting our hypothesis of a different stage of maturation between the two populations. For the ICV transplant, we also included in the analysis endogenous microglia retrieved from busulfan-treated mice injected ICV with PBS (sham-control), thus excluding that the procedure per se could significantly affect the gene expression profile of resident microglia. In this regard, it is noteworthy that some differences were observed between microglia cells retrieved from naïve untreated mice and transplanted mice (both host and repopulating cells). We believe that such differences might be associated with a response to the conditioning regimen, being this the only factor that could account for difference between endogenous microglia from untreated vs transplanted animals.

In regard to the 5xFAD data, it is unclear why mock and GFP conditions were combined. These should not be plotted together.

R: We are grateful to the Reviewer for raising this observation as we recognize that the combination of mock and GFP conditions in 5xFAD data presentation may have caused some confusion. These groups were generated to compare the TREM2-treated groups with control animals that had received an identical treatment, except from TREM2, consisting of four doses of Busulfan (25 mg/kg) and ICV transplantation of either mock-transduced HSPCs or cells transduced with a reporter vector for tracking. Being GFP a neutral cassette in terms of AD, we combined the two groups. To avoid any misunderstanding, we specified the rationale of combining data from these two conditions in the manuscript (rows 354-357).

Additional support for the role of plaque-associated cells could be added by the use of activation-associated markers, such as CD68 or CLEC7A.

R: We appreciate the Reviewer's suggestion to incorporate in our analysis of transplant-derived myeloid cells activation-associated markers, which could provide further understanding of the activity of plaque-associated cells. Thus, recognizing the importance of these markers in elucidating the role of microglia and other immune cells in the context of AD pathology, we included an assessment of CD68 in the revised version of the manuscript.Interestingly, donor-derived microglia like cells were identified by WPRE ISH probe positivity and Iba1 staining in the proximity of amyloid plaques in the TREM2-treated mice (see new Supplementary Figure 5 and results section, rows 399-406). These cells showed an activated morphology (increased soma size and thickening of processes) and robust expression of CD68, allowing to hypothesize for them an active role in reducing the amyloid burden. On the contrary, microglia distant from plaques showed in general less CD68 expression and showed morphological features characteristic of a more homeostatic/resting state.

It is also worth noting that the 5xFAD model is a model of amyloid pathology, not of Alzheimer's disease as it does not develop tau pathology or marked neurodegeneration. Furthermore it is a model that expresses two transgenes with 5 mutations, not 5 transgenes as stated in line 311.

We thank the Reviewer for noticing these errors that have been corrected in the revised text (rows 332-333).

Bennett, F. C., M. L. Bennett, F. Yaqoob, S. B. Mulinyawe, G. A. Grant, M. Hayden Gephart, E. D. Plowey and B. A. Barres (2018). "A Combination of Ontogeny and CNS Environment Establishes Microglial Identity." Neuron **98**(6): 1170-1183 e1178.

Bruttger, J., K. Karram, S. Wortge, T. Regen, F. Marini, N. Hoppmann, M. Klein, T. Blank, S. Yona, Y. Wolf, M. Mack, E. Pinteaux, W. Muller, F. Zipp, H. Binder, T. Bopp, M. Prinz, S. Jung and A. Waisman (2015). "Genetic Cell Ablation Reveals Clusters of Local Self-Renewing Microglia in the Mammalian Central Nervous System." Immunity **43**(1): 92-106.

Capotondo, A., R. Milazzo, J. M. Garcia-Manteiga, E. Cavalca, A. Montepeloso, B. S. Garrison, M. Peviani, D. J. Rossi and A. Biffi (2017). "Intracerebroventricular delivery of hematopoietic progenitors results in rapid and robust engraftment of microglia-like cells." Sci Adv **3**(12): e1701211.

Cronk, J. C., A. J. Filiano, A. Louveau, I. Marin, R. Marsh, E. Ji, D. H. Goldman, I. Smirnov, N. Geraci, S. Acton, C. C. Overall and J. Kipnis (2018). "Peripherally derived macrophages can engraft the brain independent of irradiation and maintain an identity distinct from microglia." J Exp Med **215**(6): 1627-1647.

Lund, H., M. Pieber, R. Parsa, J. Han, D. Grommisch, E. Ewing, L. Kular, M. Needhamsen, A. Espinosa, E. Nilsson, A. K. Overby, O. Butovsky, M. Jagodic, X. M. Zhang and R. A. Harris (2018). "Competitive repopulation of an empty microglial niche yields functionally distinct subsets of microglia-like cells." Nat Commun **9**(1): 4845.

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

The authors have nicely replied to previous reviewer comments. I appreciate the authors transparency and explanation on the issue of combining mock/GFP control groups in Figure 5. In the interest of transparency for the reader, I would recommend changing the graph format from box-whisker to box-whisker with the individual data points with distinct points for the mock and GFP groups. This would likely alleviate any specter of doubt from the reader as to the prudence of combining these groups.

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R: We appreciate the Reviewer's suggestion. We modified Fig.5 accordingly. In particular, we converted box-whisker of the Fig.5 (G, J, K and L) into the graphs reported, showing individual data points, distinguishing the mock and GFP groups. Data from mock and GFP mice are indicated by clear versus green dots, respectively. This information was included in the corresponding figure legend and in the related Source data file.
We adopted the same approach in graph shown in the new Supplementary Figure S3.