Engrafted parenchymal brain macrophages differ from microglia in transcriptome, chromatin landscape and response to challenge

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Microglia are yolk sac-derived macrophages residing in the parenchyma of brain and spinal cord, where they interact with neurons and other glial. After different conditioning paradigms and bone marrow (BM) or hematopoietic stem cell (HSC) transplantation, graft-derived cells seed the brain and persistently contribute to the parenchymal brain macrophage compartment. Here we establish that graft-derived macrophages acquire, over time, microglia characteristics, including ramified morphology, longevity, radio-resistance and clonal expansion. However, even after prolonged CNS residence, transcriptomes and chromatin accessibility landscapes of engrafted, BM-derived macrophages remain distinct from yolk sac-derived host microglia. Furthermore, engrafted BM-derived cells display discrete responses to peripheral endotoxin challenge, as compared to host microglia. In human HSC transplant recipients, engrafted cells also remain distinct from host microglia, extending our finding to clinical settings. Collectively, our data emphasize the molecular and functional heterogeneity of parenchymal brain macrophages and highlight potential clinical implications for HSC gene therapies aimed to ameliorate lysosomal storage disorders, microgliopathies or general monogenic immuno-deficiencies.
Macrophages were shown in the mouse to arise from three distinct developmental pathways that differentially contribute to the respective tissue compartments in the embryo and adult. Like other embryonic tissue macrophages, microglia first develop from primitive macrophage progenitors that originate in the mouse around E7.25 in the yolk sac (YS), are thought to be independent of the transcription factor (TF) Myb, and infiltrate the brain without monocytic intermediates.1–3. YS macrophage-derived microglia persist throughout adulthood. Most other tissue macrophages are however replaced shortly after by fetal monocytes that derive from myb-dependent multipotent erythro-myeloid progenitors (EMP) that also arise in the YS, but are currently thought to be consumed before birth. Starting from E10.5, definitive hematopoiesis commences with the generation of hematopoietic stem cells (HSC) in the aorto–gonado–mesonephros (AGM) region. HSC first localizes to the fetal liver but eventually seeds the bone marrow (BM) to maintain adult lymphoid and myeloid hematopoiesis. Most EMP-derived tissue macrophage compartments perseverse throughout adulthood, albeit with delay.24 Of note, replacement of YS-derived microglia and BM graft-derived parenchymal brain macrophages. Using RNaseq and ATACseq of host and engrafted macrophages isolated from mouse BM chimeras, we show that these cells acquire microglia characteristics such as longevity, morphology, and gene expression features, but still remain significantly distinct with respect to transcriptomes and chromatin accessibility landscapes. Furthermore, host and graft cells display discrete responses to challenge by peripheral endotoxin exposure. Finally, by extending our finding to clinical settings, we confirm that in human HSC transplant patients, grafted cells also remain distinct from host microglia. Collectively, these data establish that engrafted macrophages differ from host microglia even after prolonged residence in the brain parenchyma and could have considerable clinical implications for patients treated by HSC gene therapy.

Results

Engrafted brain macrophages acquire longevity and radio-resistance. Following total body irradiation (TBI), myeloid precursors enter the brain and contribute to the parenchymal macrophage compartment.26–28 Host microglia are relatively radio-resistant and unless combined with conditioning, engraftment of the brain macrophage pool was therefore reported to be limited.29–31 It furthermore remained unclear to what extent graft-derived cells acquire over time microglia characteristics, such as longevity and radioresistance. To address this issue, we generated BM chimeras by lethally irradiating WT mice (CD45.2) (950 cGy) and transplanting them with CX3CR1GFP BM (CD45.1) (Supplementary Fig. 1a). Four months after irradiation and BM transfer (BMT), monocyte precursors in the BM and circulating blood monocytes of the chimeras were all CD45.1+ and hence exclusively derived from the BM graft (Supplementary Fig. 1b, c). Analysis of the CD45int CD11b+ Ly6C–Ly6G– microglia compartment of the chimeras revealed the presence of GFP+ graft- and GFP–host-derived cells (Supplementary Fig. 1d). In line with earlier reports, grafted cells initially constituted only a small fraction of the parenchymal brain macrophage population. However, the cells progressively replaced the host microglia (Supplementary Fig. 1d). The expansion of the GFP+ infiltrate could indicate ongoing peripheral input. Alternatively, the undamaged nonirradiated CNS-resident graft-derived cells might have an advantage over the irradiated host microglia, and gradually outcompete the latter during the reported infrequent microglial local proliferation.33,34 To distinguish between these options, we performed a tandem engraftment. Recipient mice (CD45.1/2) were irradiated twice, 15 weeks apart, followed by engraftment with CX3CR1GFP BM (CD45.1) and CX3CR1Cre:R26-RFP+/+ BM (CD45.2), respectively (Fig. 1a). Blood analysis of the chimeras 7 weeks after the second BMT, showed that monocytes were exclusive derivatives of the second graft (Fig. 1b), as were myeloid BM precursors (Supplementary Fig. 1e). In contrast, analysis of the CNS compartment of the chimeras revealed the presence of three distinct macrophage populations: host microglia (CD45.1/2+), cells derived from the first graft (CD45.1+ GFP+), and cells derived from the second BM graft (CD45.2+ RFP+) (Fig. 1c, d, Supplementary Fig. 1f). The presence of cells derived from the first graft establishes that these (1) acquired radioresistance and (2) persisted in the chimeras for more than 2 months without contribution from the periphery. Corroborating the above results, cells derived from both grafts expanded on the expense of the host microglia (Fig. 1d). CX3CR1GFP (CD45.1) and CX3CR1Cre:R26-RFP+/+
(CD45.2) cells with ramified microglial morphology were detected in the brain parenchyma (Fig. 1e). Collectively, these data establish that engrafted cells adopt microglia characteristics, such as relative radioresistance, longevity, and morphology.

**Macrophages engrafting conditioned brains display expansion.**

To further characterize the engraftment process, including clonal dynamics of the BM-derived cells, we used two complementary approaches, comprising (1) transplantation of BM isolated from Cx3cr1CreER:R26Confetti (“Microfetti”) mice34 (Fig. 2a) and (2) transplantation of lineage-negative BM cells marked by a genetic barcode prior to transplantation35 (Supplementary Fig. 2a).

**Fig. 1** Engrafted brain macrophages accumulate over time and self-maintain. a Schematic of tandem BM transfer protocol. b Flow cytometric blood monocyte analysis of chimera 16 weeks post second transplantation. c Flow cytometric analysis of myeloid brain cells 16 weeks post second BMT revealing host microglia (CD45.1/2+, blue), cells derived from the first graft (CD45.1+ GFP+, red) and cells derived from the second graft (CD45.2+ RFP+, orange). d Distribution of host and graft-derived cells out of the total Ly6C/G (Gr1)–CD45lo CD11b+ cells in brain and spinal cord at two time points. Data are a summary of six mice. e Histological analysis 7 weeks post second BMT revealing ramified GFP+ and RFP+ cells with microglia morphology (scale bar 20 μm). Representative picture.
provide evidence of local proliferation, since slow-renewing cortical microglia in ‘Microfetti’ brains do not form clones over 36 weeks.34.

For the genetic barcoding procedure, lineage-negative BM cells isolated from male donors (CD45.1) were transduced with lentiviral vectors harboring advanced genetic barcodes (BC32) and a BFP reporter gene 35,36. Transduced HSC/HPCs were transferred into 8-week-old female recipient mice (CD45.2) that were irradiated (950 cGy) or conditioned with the alkylsulfonate busulfan (BU) (125 mg) used for myeloablation in pediatric and adult patients37, as well as preclinical mouse models38,39. Six months after transplantation, the right hemisphere of the mice was used for immunohistochemistry (eBFP, Iba-1; Fig.2k). From the left hemisphere, macrophages were sorted and analyzed for presence and complexity of barcodes using bioinformatics36. Both conditioning protocols resulted in efficient engraftment of the periphery and the microglia compartment (Supplementary Fig 2b, c), as also reflected in the numbers of clones displaying unique...
barcodes (Fig. 2, m, Supplementary Fig. 2d). Interestingly, while engrafted cells isolated from BM, blood, and spleen, and also CD45hi CNS cells representing hematopoietic non-microglial cell, displayed mainly shared clones, the majority of clones identified among brain macrophages of busulfan-conditioned animals did not have counterparts in other tissues and were private (Fig. 2n). Corroborating our above results and that of others,31,46 this suggests that a major fraction of the grafted cells originates from transduced precursors that seed the host CNS early after engraftment and is maintained by local proliferation independent from ongoing hematopoiesis. Taken together, these results establish that efficiently engrafted donor cells adopt the phenotype and distribution of resident microglia within this cellular network and expand locally with kinetics specific to brain regions and conditioning paradigms.

Engrafted cells have transcriptomes distinct from microglia.

BM graft-derived parenchymal brain macrophages acquire characteristics such as ramified morphology, longevity, and radioresistance and can hence be considered engrafted microglia-like cells that could potentially be employed for therapeutic purposes. Recent studies have highlighted the impact of the tissue environment on macrophage identities, including epigenomes and expression profiles.10,11 To test whether graft-derived microglia acquire in the CNS such a global molecular imprint, we transplanted lethally irradiated 6-week-old WT mice with congenic WT BM harboring CD45.1 alleles. Nine months post-transplantation, chimeras were sacrificed and brain macrophages were isolated for transcriptome and epigenome analysis by RNAseq41 and ATACseq42, respectively. At this time point, half of the CNS macrophages of the chimeras were of graft origin (Fig. 3a, Supplementary Fig. 4a).

Global RNAseq analysis of parenchymal host and graft brain macrophages isolated from individual BM chimeras revealed that engrafted cells and host microglia showed significant transcriptome overlap, clearly distinguishing them from monocytes that served as reference for a HSC-derived cell population43 (Fig. 3b, c). Of the total 11,614 detected transcripts, 10,635 (91%) displayed a lower than twofold difference between the engrafted cells and host microglia. On the other hand, 979 transcripts were differentially expressed between these two populations (absolute value of log2-fold change >1, p-value < 0.05) (Supplementary Fig. 5a). Expression of 469 genes was restricted to host microglia, while 510 genes were uniquely expressed by the engrafted cells (Supplementary Fig. 5a, b). Engrafted macrophages, but not host microglia displayed, for instance, mRNA encoding CCR2, lysozyme, CD38, CD74, Mrc1, ApoE, and Ms4a7 (Fig. 3c, Supplementary Fig. 5b). Differentially expressed genes included transcription factors (TFs) such as the basic helix–loop–helix TF Hes1, the Krueppel-like zinc finger TF Klf12, the retinoic acid receptor Rxrα, and the TGFβ-associated signal transducer Smad3, were preferentially transcribed in host cells. Conversely, engrafted macrophages displayed increased expression of the estrogen receptor Esr1, the runt-domain TF Runx3, and the macrophage survival factor Nr4a1, as compared to host cells (Fig. 3d). Of note, the host microglia in this case were irradiated and differences observed between the two populations could hence arise from radiation damage; transcriptomes of engrafted cells however also differed significantly from age-matched non-irradiated microglia (Supplementary Fig. 5c).

Supporting the notion of their microglia-like identity, engrafted cells expressed similar levels of the DNA–RNA binding protein TDP-43 encoded by the Tardbp gene recently implied as a regulator of microglial phagocytosis,44 the two-pore domain potassium channel THIK-1, encoded by the Kcnk13 gene, and shown to be critical for microglial ramification, surveillance, and IL-1β release45 and the TF Meg3, reported to restrain microglia responses.46 (Fig. 3f) Likewise, the grafted also displayed expression of microglia signature genes that have been proposed to distinguish these cells from other tissue macrophages and acute monocyte infiltrates associated with inflammation,47,48 including Fc receptor-like molecule (Fcrls) and TGFβ receptor (Tgfb), which is critical to establish microglia identity.47 (Fig. 3f). Other proposed microglia signature genes, such as P2ry12, Tmem119, SiglecH, and HhexB displayed either significantly reduced expression in the grafted cells or were exclusively expressed by host microglia, like the ones encoding the sodium/glucose cotransporter 1 (Slc2a5), the phosphoglycoprotein protein CD34 (Cd34), and the transcriptional repressors Sall1 (Sall1) and Sall3 (Sall3) (Fig. 3g, Supplementary Fig. 5d). Of note, lack of some microglia markers had been reported before for cells retrieved from acutely engrafted brains.47,49,50.

The expression signature of the engrafted macrophages showed a considerable overlap with the transcriptome of perivascular macrophages,51–53 including present and absent transcripts, such as ApoE, Msr4a7, Slc2a5, and Sall1, respectively. Gene set enrichment analysis (GSEA)52 revealed that engrafted macrophages displayed an activation signature as compared to host microglia (Supplementary Fig. 6a). Finally and corroborating our data, the list of genes we report as differentially expressed by engrafted and host cells also displayed a considerable overlap with results recently reported by two other groups.54,55 (Supplementary Fig. 6b).

Sall1, a member of the Spalt ("Spalt-like" (Sall)) family of evolutionarily conserved transcriptional regulators critical for organogenesis, acts as a repressor by recruitment of the nucleosome remodeling and deacetylase corepressor complex (NurD). Binding motifs of Sall1 and hence its direct genomic
targets remain undefined precluding a direct assessment of the impact of the lack of the repressor on the expression signature of the grafted macrophages. Interestingly though, comparison of the recently reported list of genes differentially expressed by WT and Sall1-deficient microglia50 and that of host and graft brain macrophages revealed in this study, showed significant overlap (Fig. 3h). This included expression of genes otherwise restricted to macrophages residing in non-CNS tissues, such as Msr1, encoding a scavenger receptor, and Cyp4f18 encoding cytochrome P450 (Fig. 3i). Furthermore, like Sall1-deficient microglia50, grafted CNS macrophages displayed an activation signature, as reflected by expression of Cybb encoding the Cytochrome b-245 heavy chain and Axl encoding a member of a tyrosine kinase receptor family critical for debris clearance20 (Fig. 3i,
Fig. 3 Comparative transcriptome analysis of grafted cells and host microglia. a Gating strategy for isolation of CNS macrophages. Host microglia were defined as Ly6C+/CD11b−/CD45.2+ cells; graft-derived cells were defined as Ly6C+/CD11b+CD45.1+. b Principal component analysis of transcriptomes of engrafted cells and host microglia and transcriptomes of monocytes subsets46. Source data are provided as a Source Data file. c Heatmap of RNA seq data of engrafted cells and host microglia compared to transcriptomes of Ly6C− and Ly6C+ monocyte subsets46. Analysis was restricted to genes, which showed a twofold difference and yielded p-value < 0.05 between at least two populations. Source data are provided as a Source Data file. d Heatmap showing differential TF expression profiles of engrafted cells and host microglia. Source data are provided as a Source Data file. e Examples of significantly differential (log2FC>1 and p-value < 0.05) gene expression enriched in engrafted cells. Graphs show normalized reads from RNA seq data of samples acquired in (a), n = 4. f Examples of genes expressed in similar levels in host and engrafted cells. Graphs show normalized reads from RNA seq data of samples acquired in (a), n = 4. None of the genes were differentially expressed (log2FC < 1). Tgfr1 and Kcnk13 were of low significance (p-value < 0.05) but did not meet our FC threshold. Examples of significantly differential (log2FC > 1, p-value < 0.05) gene expression enriched in host microglia. Graphs show normalized reads from RNA seq data of samples acquired in (a), n = 4. h Venn diagram showing overlap of genes differentially expressed by WT and Sall1-deficient microglia53, and genes differentially expressed by host and engrafted brain macrophages. i Examples of gene expression of engrafted and host cells of genes expressed in non-CNS macrophages or Sall1-deficient microglia. Source data are provided as a Source Data file.

Supplementary Fig. 6b). This suggests that a major fraction of the differential expression of host microglia and engrafted cells could be explained by the specific absence of the transcriptional repressor Sall1 from the former cells. Overall, these findings establish that engrafted macrophages that persist in the brain and acquire microglia characteristics such as morphology and radioresistance, also show significant transcriptome overlap with host microglia, but remain a molecularly distinct population.

Distinct chromatin accessibility landscapes in grafted and host cells. To further define engrafted cells and host microglia, we performed an epigenome analysis using ATACseq that identifies open chromatin regions by virtue of their accessibility for “tagmentation” by transposases42. Correlated ATACseq replicates (Supplementary Fig 7a) performed on graft and host microglia isolated from BM chimeras detected 58,947 total accessible regions (corresponding to 16,156 genes). Corroborating the observed differential gene expression (Fig. 3), host microglia but not engrafted cells, displayed ATAC signals in the Sall1 and Klf2 loci, as indicated in Integrative Genomics Viewer (IGV) tracks (Fig. 4a). ATAC peaks in other genomic locations, such as ApoE and Ms4a7, were restricted to genomes of engrafted cells, in line with mRNA detection in these cells, but not host microglia (Fig. 4b). As ATACseq does not discriminate between bound transcriptional activators and repressors, some differentially expressed loci did not show epigenetic differences. This included for instance the MHC II locus (H2-ab1), which displayed similar ATACseq peaks in host microglia and the graft that lack and display H2-ab1 transcripts, respectively (Fig. 4c). These loci might be transcriptionally silenced, but activated upon cell stimulation. Similar “poised” states, that might be revealed following challenge, can be assumed for gene loci, that displayed differential ATAC peaks, but were transcriptionally active in neither the host nor the engrafted cells. Finally, rare genes, such as the Dbi locus showed equal expression, but differential ATAC profiles suggest that their transcription is driven by distinct TFs (Fig. 4c). Global quantification of differential ATAC peaks between the two brain macrophage populations revealed that 6% of the total accessible regions (or 8.7% of the associated genes) were distinct. Specifically, 1506 peaks (corresponding to 941 genes) displayed a >4-fold significant (p-value < 0.01) enrichment in host microglia and 2176 peaks (corresponding to 1465 genes) were increased in BM graft-derived cells (Fig. 4d).

To identify potential TFs responsible for the differential transcriptomes and chromatin accessibility landscapes of engrafted and host microglia, we applied a TF Binding Analysis (TBA) machine learning model56. TBA learns to jointly weigh 100s of motifs drawn from the JASPAR and CISP databases to distinguish open chromatin sites from GC-matched genomic background. By examining the contribution of each motif to the model’s performance, TBA assigns a significance level to each motif. This analysis revealed a class of highly significant motifs correlated with open chromatin that were common to both host and graft cells and a variety of other myeloid cells (p < 10e−20) such as CTCF, SPI1, and Runx binding motifs (Supplementary Fig. 7b). Of the more intermediately ranked motifs, 41 motifs were more important in either host or graft cells as measured by the log-likelihood ratio (LL) when comparing graft and host microglia (LL > = 10e−2, blue points Fig. 4e). Ten motifs were preferentially detected in host microglia (Fig. 4e), including motifs for TFs such as IRF8, which is a critical regulator of microglia identity37, as well less characterized motifs such as the C2H2 zinc finger motif. The latter motif could potentially be recognized by Sall1, which contains tandem C2H2 zinc fingers. Motifs preferentially detected in grafted cells included CEBPα, RFX, and Jun motifs (Fig. 4e). Motifs that were preferentially detected in either graft or host cells exhibited even greater differences in comparison to other myeloid cell populations, consistent with environmental cues directing chromatin remodeling of grafted cells (Fig. 4f). Collectively, these observations substantiate the conclusion that while grafted cells adopt epigenetic characteristics similar to microglia, they remain distinct from host cells even after prolonged CNS residence.

Engrafted macrophages respond unlike microglia to challenge. Given the significant transcriptome and chromatin landscapes differences between the host and BM graft-derived macrophages that persists for extended periods of time post transplantation, we next examined whether the two populations are functionally distinct. To that end, chimeras were challenged 9 months post-transplantation by a peripheral injection of the bacterial endotoxin lipopolysaccharide (LPS), an established paradigm for inflammation associated with robust microglia responses to systemic cytokine secretion. Host and engrafted cells were isolated from the brains of the chimeras 12 h post LPS challenge, global RNA and ATAC sequencing were performed, and the results were compared to the samples obtained from non-challenged mice presented earlier (Figs. 3, 4). Principal component analysis (PCA) revealed a high degree of similarity within each group, but segregation of the host and graft samples (Fig. 5a). Host and grafted cells responded with altered expression of 745 shared genes. In total, 940 genes were changed in grafted cells only, and 602 genes were changed in host microglia upon LPS challenge (Fig. 5b). Examples for these three categories are shown in Fig. 5c, d and Supplementary Fig. 8a. Genes commonly induced by engrafted cells and host microglia in response to the LPS challenge comprised Tnf, Ccl5, and Tnfαp3, encoding the A20 deubiquitinase that negatively regulates NF-κB-dependent gene expression. Commonly downregulated genes comprised Trem2, Cx3cr1, and Aif1. The graft-specific response included...
Fig. 4 Comparative epigenome analysis of grafted cells and host microglia. 

a. IGV tracks of Sall1 and Klf12 loci showing ATAC signals in host (red) but not engrafted (blue) cells. \( N = 3 \).

b. IGV tracks of ApoE and Ms4a7 loci showing ATAC signals in engrafted cells (blue) but not host (red) microglia. \( N = 3 \).

c. ATAC-seq IGV tracks (left, \( n = 3 \)) and normalized RNA seq reads (right, \( n = 4 \)) of H2-ab1 and Dbi in host microglia (red) and engrafted cells (blue). +—p-value < \( 10^{-5} \), n.s.—p-value > 0.05. 

d. Analysis of all 58,947 detected ATAC peaks, from which 1506 peaks and 2,176 peaks displayed >4-fold significant (p-value < 0.01) enrichment in host microglia and engrafted cells, respectively. Source data are provided as a Source Data file.

e. Comparison of motif significance in resting host and grafted cells. P-values were calculated using TBA models trained on intergenic peaks from host and grafted cells. Significant motifs that show a large difference (p < 10e−5, log-likelihood ratio > 2) are indicated in blue points. Source data are provided as a Source Data file.

f. Heatmap of the significance of motifs in various myeloid cell types and host cells. The intensity of the color indicates greater significance (−log10 p-value) of each motif. Source data are provided as a Source Data file.
upregulation of Clec4e, Pirb, Isg15, and Irf7 and downmodulation of Mgl2, Cmah, and CD36. Genes specifically induced in host microglia encoded the scavenger receptor Marco, Gpr65, Tlr2, and Il12b. Moreover, host microglia silenced the expression of Sall1 and Upk1b (Fig. 5d). Ingenuity analysis of transcriptomes of engrafted and host brain macrophages isolated from chimeras with and without peripheral LPS challenge revealed potential distinct upstream regulators acting on these populations, as well as a differential representation of activated functional pathways (Supplementary Table 1). Engrafted macrophages displayed for instance activation of pathways controlled by IL1b and Ifng and suppressed by IL-10.

ATACseq analysis revealed differential epigenome alterations between engrafted and host cells in response to the LPS challenge. Specifically, correlated ATACseq replicates (Supplementary Fig 9a) performed on engrafted and host cells isolated from BM
Engrafted macrophages in human brain differ from microglia.

Engrafted brain macrophages differ from host microglia by their gene expression (Fig. 3b). To confirm this finding for protein expression, we performed a histological analysis of brains of the BM chimeras generated by TBI and BU conditioning (Supplementary Fig. 2d). Engrafted cells and host microglia were identified by IBA-1 staining. Graft-derived cells were defined according to eBFP expression conveyed by the lentiviral construct (Fig. 2k). In concordance with the transcriptome data (Fig. 3g), analysis for Tmem119 and P2ry12 expression revealed the absence of these markers from the graft (Fig. 7a, b), while eBFP+ cells displayed ApoE and MHC class II (Supplementary Fig. 10).

To finally extrapolate our finding to a human setting, we analyzed post mortem brains of patients who underwent HSC transplantation. Specifically, we took advantage of gender-mismatched grafts that allowed identification of the transplant by virtue of its Y-chromosomes through chromogenic in situ hybridization (CISH). Ramified IBA-1+ microglia-like cells harboring the Y chromosome could be readily identified juxtaposed to Y chromosome-negative host microglia in cortex, cerebellum, and hippocampus sections of the subject brains (Fig. 7c). Expression of the purinergic P2Y12 receptor has been proposed earlier to serve as marker for human microglia. Moreover, absence of the P2Y12 receptor compromises microglial activation by nucleotides and could thus have functional implications. As observed in the murine chimeras, the human P2Y12 receptor expression was found to be restricted to host cells, but absent from Y chromosome-positive brain macrophages cells (Fig. 7d). Collectively, these data establish that in the CNS of patients who underwent HSC transplantation, graft-derived cells remained functionally distinct from host microglia and strengthen the conclusion that HSC-derived engrafted cells differ from host microglia.

Discussion

Here we established that BM-derived brain macrophages that persistently seed the CNS of recipient organisms following irradiation or myeloablation remain distinct from host microglia with respect to their transcriptomes, chromatin accessibility landscapes, and response to challenge.

Following the engraftment of conditioned recipient mice, transplanted cells establish under the influence of the CNS microenvironment, a characteristic microglia transcriptome that distinguishes these cells from other tissue macrophages. Thus, nine-tenth of their transcriptome is shared with host microglia, including expression of Fc receptor-like molecules (Fcrls) and Tgb receptor (Tgbr), as well as the MADS box transcription enhancer factor 2 (Mef2c). Moreover, residence in the CNS endowed engrafted cells with microglia characteristics, such as longevity, radio-resistance and ramified morphology. Engrafted cells however failed to adopt complete host microglia identity even after prolonged CNS residence. Corroborating and extending earlier reports, this included significantly reduced mRNA expression of the microglia markers Tmem119, SiglecH and P2yr12 and complete lack of the transcriptional repressors Sall1 and Sall3. Conversely, engrafted macrophages expressed genes absent from host microglia, including Ccr2, Ifnar1, Ms4a7 and ApoE, and related to the absence of Sall1. Transcriptomes of engrafted macrophages showed considerable overlap with perivascular macrophages and indication of cell activation, such as an underrepresentation of a regulatory pathway driven by IL-10, as compared to host microglia. Comparative transcriptome analysis showed that our data are well in line with recent studies that reported that also macrophages that engraft the brain of mice conditionally depleted of microglia due to a Csf1r deficiency retain a transcriptional identity distinct from host cells.

Analysis of open chromatin revealed that graft cells acquire a transposase-accessible profile that is similar to that of host cells and is enriched for a common set of motifs that are recognized by TFs known to be important for microglia development, such as PU.1 and MEF2c. However, host and graft cells also exhibit...
Fig. 6 Comparative epigenome analysis of graft and host microglia post LPS challenge. a Analysis of all 46,485 detected ATAC peaks, from which 552 peaks and 841 peaks displayed >4-fold significant (p-value < 0.01) enrichment in host microglia and engrafted cells isolated from challenged mice, respectively. b Comparison of motif significance in activated/challenged host and grafted cells. P-values were calculated using TBA models trained on intergenic ATAC-seq peaks from host and grafted cells. Significant motifs that show a large difference (p < 10e−5, log-likelihood ratio > = 2) are indicated in blue points. Motif logos visualizing the position frequency matrix of NF-kappaB and AP-1 motifs are annotated. c Challenge induced alterations in Marco locus. Normalized sequence reads of Marco mRNA in engrafted cells and host microglia isolated from LPS challenged and unchallenged BM chimeras (top); normalized ATACseq profiles of Marco locus (bottom), with enlarged areas highlighting induced ATACseq peaks and predicted motifs. d Challenge induced alterations in Spp1 locus. Normalized sequence reads of Spp1 mRNA in engrafted cells and host microglia isolated from LPS challenged and unchallenged BM chimeras (top); normalized ATACseq profiles of Spp1 locus (bottom), with enlarged areas highlighting induced ATACseq peaks and predicted motifs. Source data are provided as a Source Data file.
significant differences in open chromatin that are associated with distinct motif enrichment patterns and the observed differences in gene expression. These findings imply that the distinct developmental origins of host and graft cells determine the ability of the brain environment to fully activate the complement of transcription factors required for microglia identity, most notably exemplified by lack of induction of *Sall1* in graft cells. The differences in chromatin landscapes under resting conditions are likely to contribute to the host and graft-specific responses to LPS challenge. This possibility is supported by the observation that
alternative motifs for NFκB and AP-1 factors are enriched in open chromatin of host and graft cells. We interpret this finding to reflect the binding of NFκB and AP-1 dimers and heterodimers to different locations in the genome that are specified by the specified by host or graft-specific combinations of transcription factors, respectively.

The exact origin of the BM-derived engrafted cells in the chimeric organisms remains to be defined. In a classic study, Ajami and colleagues established that non-parenchymal brain macrophages that can persistently seed the host brain originate from non-monocytic BM-resident myeloid progenitors characterized by the absence of C/EBPε expression26. Likewise, other studies suggested that a transient wave of early hematopoietic progenitors infiltrates the host CNS during transplantation and following local proliferation, establishing the graft31. This notion is supported by the results of our ‘Microfetti’ and barcoding approaches that establish that engrafted macrophages undergo clonal proliferation and thereby likely progressively outcompete irradiation or busulfan-damaged host microglia. Moreover, the conclusion that engrafted cells arose from cells that do not contribute to long-term hematopoiesis in the chimeras is also in line with the prominent detection of private clones in this population, which are not shared with the other hematopoietic compartments.

Future studies could aim to identify cells that upon engraftment will give rise to closer mimics of host microglia, including for instance expression of Sall1. This could include cells linked with the unique developmental YS origin of microglia34, or otherwise manipulated cells such as microglia-like cells derived from induced-pluripotent-stem-cell (iPS)-derived primitive macrophages61. Furthermore, in the context of gene therapy, viral vectors could be used to express transgenes to engineer the engrafted cells to boost engraftment and modulate their function. Of note however, under certain pathological conditions the distinct engrafted BM-derived macrophages we report might also be advantageous as compared to host microglia. BM-derived cells could for instance be superior to YS-derived microglia in the handling of the debris burden associated with senescence62 or amyloid plaques that arise during Alzheimer’s disease63. Eludiation of such scenarios should profit from the molecular definition of the engrafted cells and host microglia like the one provided in this study. However, in vivo functions of microglia remain poorly understood and future dedicated experimentation will be required to compare the performance of engrafted macrophages and host microglia in different disease models during aging and specific challenges.

Recent studies revealed a signature of disease associated microglia, termed ‘DAM’32 or ‘MGnD’64, that is induced by various brain-intrinsic changes in the absence of massive peripheral infiltrates, though not following peripheral LPS challenge58,65, and can occur on acute to chronic time scales56. Of note, engrafted brain macrophages displayed robust constitutive expression of some of these DAM genes, such as ApoE and Axl. Likewise, as opposed to microglia58, engrafted BM-derived brain macrophages responded to the LPS challenge with the induction of DAM/ MGnD hallmarks, such as the CD44 ligand Spp1/ Osteopontin. The latter might have implications when considering brain macrophage contributions to CNS pathologies. Moreover, our data support the notion that expression of genes included in the DAM/MGnD signature are in microglia under stringent control, potentially including repression by Sall1.

Results obtained from fate mapping models currently suggest that at least in the brain of unchallenged C57BL/6 mice kept in specific-pathogen-free (SPF) facilities, parenchymal macrophages are exclusively comprised of YS-derived microglia. Further experimentation will however be required to assess how absolute this exclusion of HSC-derived macrophages is, in particular following challenges. Moreover, it remains currently unclear to what extent HSC-derived macrophages might be able to seed the human brain, i.e. during extended aging, and could hence impact brain pathologies.

Tissue macrophages, such as Kupffer cells (KC) and alveolar macrophages (AM), have been reported to be faithfully replaced by BM-derived cells in irradiation chimeras and other small animal models involving deficiencies of the resident compartment6–9. While these studies were restricted to transcriptome comparison and hence might have missed epigenetic differences between graft and host cells, the inability of HSC-derived cells to achieve full host cell identity might be unique to microglia and related to features particular to these cells. Specifically, among adult tissue macrophages, only microglia derive from primitive YS macrophages and this origin could define cell identity. In contrast, generation of both KC and AM involves a monocytic intermediate, and their re-generation might hence be attainable by the closer related HSC-derived cells that can also give rise to monocytes. Alternatively, establishment of the ‘bona fide’ microglia signature might require ‘physiological’ microglia development in the developing CNS that is associated with profound transient activation of this brain macrophage compartment12,13,57,66.

Collectively, the demonstration that engrafted cells and host microglia remain distinct sheds light on the molecular and functional heterogeneity of parenchymal brain macrophages. Moreover, when extrapolated to the human setting, our findings could have major implications for patients treated by HSC gene therapy to ameliorate lysosomal storage disorders, microgliopathies or general monogenic immuno-deficiencies.

Methods

Mice. For generation of BM chimeras, wild type C57BL/6 J mice (Harlan) were used as recipients, Cx3cr1GFP or Cx3CR1Cre:R26-RFP mice32,67 were used as donors. Recipient mice were lethally irradiated with a single dose of 950 cGy using an XRAD 320 machine (Precision X-Ray (PXI)) and reconstituted the next day by i.v. injection of 5 × 106 donor BM cells per mouse. All animals bred at the Weizmann animal facility were maintained under specific pathogen-free and handled according to protocols approved by the Weizmann Institute Animal Care Committee as per international guidelines. Female C57BL/6 J wild type recipient mice (Charles River) received full body irradiation (7 Gy) in the RS 2000 Biological Research Irradiator at 8 weeks of age. After 24 h, 5 × 106 donor BM cells isolated from Cx3cr1CreERT2;R26RConfetti/’Microfetti’ mice34 were injected into the tail vein of recipients. Recipient mice received 500 μl Cotrim K-ratiopharm® antibiotics in 250 ml drinking water for 2 weeks after BM reconstitution. Tamoxifen (Sigma) dissolved in corn oil (Sigma) was subcutaneously applied in a single dose of 10 mg distributed along the flanks. Mice were maintained in specific-pathogen-free facility with chow and water provided ad libitum. Animal experiments performed in
Incubating 3 min at 70 °C in Zn2+ synthesis kit (NEB) in a 20 mL reaction, incubating for 2 h at 16 °C. The product by qRT-PCR and samples with a similar CT were pooled (up to eight samples per pool). Samples were removed with Turbo DNase I (Ambion) 15 min at 37 °C and the ampli-strand expression levels were calculated and normalized using the HOMER software (Agilent Technologies). Libraries where sequenced on the Illumina NextSeq 500 obtaining an average of 20 million reads per sample. Putative open chromatin regions (peaks) were called using HOMER(5) (using parameters compatible with IDR) with FDR cut-off between at least two populations and adjusted p value for multiple gene testing > 0.05. Gene expression matrix was clustered using k-means algorithm for a range of possible values (4 ≤ k ≤ 10). The irreproducible discovery rate (IDR) was computed for each peak using the Homer peak score for each replicate experiment (https://github.com/ubooley/idr/); peaks with IDR > 0.05 were filtered away. Normalization and differential expression analysis was done using the DESeq2 R-package. Intergenic peaks annotated by Homer were used to train TBA models for each cell type using default parameters (https://github.com/enhaintgo/tba). The significance of each motif in each cell type was assessed using the predictive performance of the trained TBA model and a perturbed model that cannot recognize one motif using the chi-squared test.

Histology. Mice were transcardially perfused with PBS followed by 4% paraformaldehyde in PBS. Mouse brains were post-fixed for 6 h or overnight at 4 °C and processed for frozen sectioning as previously detailed(24). Free floating 50-μm cryosections and 14-μm cryosections on slides were prepared from Microfetti and lineage negative barcoded BM chimeras, respectively. Tissue were permeabilized in blocking solution (0.1% Triton X-100, 5% bovine albumin, and PBS) for 2 h at room temperature and incubated overnight at 4 °C with primary antibodies: 1:500 rabbit anti-ApoE (Abcam), 1:1000 rabbit anti-TMEM119 (Abcam), 1:500 rabbit anti-P2RY12 (AnaSpec), 1:200 goat anti-APoE (Millipore), and 1:100 mouse anti-CD250 antibody (Chemicon). Antigen retrieval was performed prior to ApoE staining for 40 min at 92°C in pH 9 citrate buffer. Corresponding secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 568, or Alexa Fluor 647 (Life Technologies) were applied at 1:100 with nuclear counterstain by 4',6-diamidino-2-phenylindole (DAPI, Sigma) at 1:5000 for 2 h at room temperature. Sections were mounted in ProLong® Diamond Antifade Mountant (Life Technologies).

Motif screen with transcription factor binding analysis. We used Transcription Factor Binding Analysis (TBA) models to identify enriched motifs in open chromatin in comparison to a set of randomly selected genomic loci (matched for GC content). For each of the sequenced samples, ATAC-seq peaks and background loci, TBA calculates the highest motif score for each of the motifs (in either orientation) included in the model. The sequences of open chromatin regions and background loci as well as the corresponding motif scores are used to train the TBA model to distinguish open chromatin from background loci (using five-fold cross validation). A TBA model is trained and cross scored the putative open chromatin given a genomic sequence by computing a weighted sum over all the motif scores computed for that sequence. The weight for each motif is learned by iteratively modifying the weights (starting from random values) until the model’s ability to differentiate open chromatin from background sequences no longer improves. The significance of a given motif was assigned by comparing the predictive performance of a trained TBA model and a perturbed model that cannot recognize that motif using the likelihood ratio test. We trained TBA models for each cell type, using version 1.0 of TBA and default parameters (source code and executable files are available at: https://github.com/enhaintgo/tba). For a complete description see ref. 25.

Motif enrichment and analysis. Brain images of Microfetti and lineage negative BM chimeras were acquired on the Keyence BZ-9000 inverted microscope using a 20X/0.75 NA objective lens. Images were processed and analyzed using ImageJ (NIH). Intercellular distances were determined by Calculate-NearestNeighbor from Kota Miura (https://doi.org/10.5281/zenodo.1323726). Cell morphological analyses were performed with ImageJ plugins Simple Neurite Trace(26) and Sholl Analysis(27) using default settings.

Human gender-mismatched stem cell transplantation experiments. Experiments on human tissue samples were performed according to the Declaration of Helsinki. Ethical approval was obtained from the local Research Ethics Committee.
Committee of the University Medical Center of Freiburg (ref. no. 10008/09).

The analyzed brain tissue samples of female patients, who underwent gender-mismatched transplantation from a peripheral blood stem cell transplantation (PBSC-T) donor, were dissected and embedded in parafﬁn. Brain regions including the temporal cortex, hippocampus, and cerebellum were produced by an in-house R-script using the VennDiagram package.

Combined immunohistochemistry and in situ hybridization. After the death of the two patients, brains were transferred into 4% paraformaldehyde (PFA) within 48 h and ﬁxed for at least 3 weeks. After ﬁxation, representative tissues from several brain regions including the temporal cortex, hippocampus, and cerebellum were dissected and embedded in parafﬁn. Routine neuropathological examination revealed minimal signs of neurodegeneration in Patient 1, according to Braak stage I-III. Patient 2 showed no signs of brain pathology. Combined immunohistochemistry (IHC) and chromogenic in situ hybridization (CISH) were performed on 10 µm thick sections. Sections were deparafﬁnized and heated at 92 °C, at pH 6 for 40 min for antigen retrieval. For IHC, the sections were incubated with the respective primary antibodies for 30 min at room temperature: 1:1000 rabbit anti-IBA-1 (Abcam, clone EPR16588), 1:1500 rabbit anti-P2RY12 (Sigma-Aldrich, polyclonal). Secondary goat anti-rabbit antibodies (Sudios Biotech) were applied at 1:200 for 1 h at room temperature. Liquid Permanent Red Substrate-Chromogen (Agilent Dako) was used for visualization of the antigen. After 5 min post ﬁxation in 4% PFA and rinse in deionized water, CISH was performed on the sections using the ZytoDot CISH Implementation Kit (ZytoVision) according to manufacturer’s instructions with the following modiﬁcations. After incubation in ethylene diamine tetra-acetic acid (EDTA) at 95 °C for 15 min, sections were treated with pepsin solution at 37 °C for 6 min. After stepwise dehydration, 12 µl of ZytoDot CEN Yq12 digoxigenin-linked probe (ZytoVision) was added to each brain section to bind to the Yq12 region of the human Y-chromosome for at least 20 h at 37 °C.

Following the washing and blocking steps, the sections were incubated in mouse anti-digoxigenin antibody solution, treated with horseradish peroxidase-conjugated anti-mouse antibody at 37 °C for 30 min, and bound to 3,3’-diaminobenzidine at 37 °C for 15 min. Nonspeciﬁc signals were counterstained with hematoxylin. Images were acquired using MikroCam II with a UPLAN FLN 40×/0.75 NA objective on a BX40 microscope (Olympus).

Barcode analyses. DNA was extracted from peripheral blood, bone marrow, spleen and sorted microglia and CD45+CD11b+ cells were used for barcode ampliﬁcation, multiplexing, as well as the bioinformatic processing. Unique barcodes with more than 100 reads per sample were taken into further analyses. Venn diagrams were produced by an in-house R-script using the VennDiagram package.

Digital droplet PCR. To determine the chimerism in the sorted microglia, digital droplet PCR was performed. In a duplex reaction, a Y-chromosome-speciﬁc fragment (and a control amplicon (located in the erythropoietin receptor) were simultaneously ampliﬁed and analyzed using the QX200 system (BioRad).

Statistical analysis. Mean data are shown. Mann–Whitney test, D’Agostino–Pearson test, two-tailed unpaired t test, and Welch’s t test were performed in GraphPad Prism7. Statistical signiﬁcance was taken at p < 0.05.

Data availability

The accession codes for the RNA-seq and ATACseq datasets reported in this paper can be found at GEO: GSE122789. Additional data that support the ﬁndings of this study are available as Source Data for Fig. 3b–d, h, Fig. 4d–f, Fig. 5a–d, Fig. 6a–d, Supplementary Figures 7b, 8a, and 10b. Other data are available from the corresponding author upon request.

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