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Commensal microbiota divergently affect myeloid subsets in the mammalian central nervous system during homeostasis and disease

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Abstract

The immune cells of the central nervous system (CNS) comprise parenchymal microglia and at the CNS border regions meningeal, perivascular, and choroid plexus macrophages (collectively called CNS-associated macrophages, CAMs). While previous work has shown that microglial properties depend on environmental signals from the commensal microbiota, the effects of microbiota on CAMs are unknown. By combining several microbiota manipulation approaches, genetic mouse models, and single-cell RNA-sequencing, we have characterized CNS myeloid cell composition and function. Under steady-state conditions, the transcriptional profiles and numbers of choroid plexus macrophages were found to be tightly regulated by complex microbiota. In contrast, perivascular and meningeal macrophages were affected to a lesser extent. An acute perturbation through viral infection evoked an attenuated immune response of all CAMs in germ-free mice. We further assessed CAMs in a more chronic pathological state in 5xFAD mice, a model for Alzheimer’s disease, and found enhanced amyloid beta uptake exclusively by perivascular macrophages in germ-free 5xFAD mice. Our results aid the understanding of distinct microbiota-CNS macrophage interactions during homeostasis and disease, which could potentially be targeted therapeutically.

Keywords: Alzheimer’s disease; CNS-associated macrophages; LCMV; microbiota; microglia

Subject Categories: Immunology; Neuroscience

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Introduction

As innate immune cells in the central nervous system (CNS), tissue macrophages encompass a heterogeneous population of myeloid cells. Microglia are localized in the CNS parenchyma, while the non-parenchymal CNS-associated macrophages (CAMs) reside at the border regions as choroid plexus macrophages (cpMΦ), leptomeningeal macrophages (mMΦ), and perivascular macrophages (pvMΦ) (Prinz et al., 2017; Briosi et al., 2020). Despite the fact that these macrophage subsets are generally involved in scavenging of cell debris, pathogens, and molecules, they fulfill distinct region-specific tasks, such as support of neurons and oligodendrocytes by microglia in the brain parenchyma, or vascular remodeling and drainage of toxic protein aggregates by pvMΦ (Zhou et al., 2010; Ueno et al., 2013; Hawkes et al., 2014; Faraco et al., 2016; Hagemeyer et al., 2017; Herz et al., 2017; Kierdorf et al., 2019). Recent studies have established that both microglia and CAMs are self-renewing long-living cells with a solely prenatal yolk sac origin. A notable exception is cpMΦ that is replaced by C-C chemokine receptor type 2 (CCR2)-dependent Ly6C<sup>high</sup>-expressing blood-derived monocytes over time analogous to other selected peripheral tissue macrophages (Goldmann et al., 2016; Hoeffel & Ghinhoux, 2018; Van Hove et al., 2019). Recently, it was suggested that, distinct from microglia, development of CAMs occurs independent of transforming growth factor beta (TGF-β) (Utz et al., 2020).

In the past, the discrimination of the CNS tissue macrophage subsets was mainly based on the anatomical region. In addition, some markers are specifically expressed either by CAMs or by microglia. Under homeostatic conditions, these include Mannose receptor C-type 1 (Mrc1, encoding CD206), Cd163, and Lymphatic vessel endothelial hyaluronan receptor (Lyve1) for CAMs or hexosaminidase subunit beta (Hexb) and Transmembrane protein 119...
(Tmem119) for microglia (Zeisel et al, 2015; Goldmann et al, 2016; Jordao et al, 2019; Van Hove et al, 2019; Kim et al, 2021). Common myeloid cell markers such as ionized calcium binding adaptor molecule 1 (Iba1), Cd11b, or Cx3 chemokine receptor 1 (Cx3cr1) are expressed by both microglia and CAMs (Prinz et al, 2017). Compared to microglia, the biological functions of CAMs are under-studied and not yet well understood (Kierdorf et al, 2019). It is suggested that CAM subsets are implicated in scavenging cellular debris, pathogens, and molecules (Mrdjen et al, 2018; Kierdorf et al, 2019). In mouse models of Alzheimer’s disease (AD), a critical role of pvMFΦ in amyloid beta (Aβ) clearance was reported (Hawkes & McLaurin, 2009; Mildner et al, 2011).

It is widely accepted that the microglial identity in the CNS is imprinted by ontogenetic, species- and tissue-specific as well as environmental factors, such as the gut microbiota (Gosselin et al, 2014; Lavín et al, 2014; Enry et al, 2015; Amit et al, 2016; Geirsdot tir et al, 2019; Enry & Prinz, 2020). Previously, we showed that host microbiota control maturation and function of microglia under steady-state and pathological conditions (Enry et al, 2015). Mice born and raised in a germ-free (GF) environment, constitutively lacking commensal microbes, exhibited an immature microglial phenotype with altered gene expression profile, increased cell numbers, hyper-ramified morphology, and impaired immune response compared with colonized mice raised under specific pathogen-free (SPF) conditions (Enry et al, 2015). Short-chain fatty acids (SCFAs), microbiota-derived fermentation products, were shown to mediate these effects on microglia in homeostatic and disease conditions (Enry et al, 2015; Sampson et al, 2016; Chu et al, 2019; Enry & Prinz, 2020; Mezo et al, 2020). Additionally, a microbiota-dependent Aβ uptake by microglia was shown in the 5x familial AD (5xFAD) mouse model for AD (Mezo et al, 2020). However, whether all CNS myeloid cells across the CNS are shaped by gut microbiota in the homeostatic and diseased CNS, the underlying transcriptional program on a single-cell level as well as functional implications remained unknown.

Overall, we found an altered transcriptional phenotype of GF-associated CAMs. Notably, we identified predominantly cpMFΦ as highly responsive to the GF status. Interestingly, the presence of a complex microbiota was a crucial determinant of the response of CAMs to an acute viral infection with lymphocytic choriomeningitis virus (LCMV). In a more chronic pathological state in the 5xFAD mouse model of AD, we revealed microbiota-dependent alterations in the Aβ uptake only in pvMFΦ. Our results indicate context-dependent specific effects of environmental microbes on selective CNS myeloid cells and thereby modulating CNS immunity.

**Results**

**scRNA-seq identifies microbiota-dependent imprints on myeloid subsets in distinct CNS compartments**

To assess myeloid cells at single-cell level, we dissected several CNS compartments. Viable CD11b⁺CD45⁺CD3⁻B220⁻Ly6G⁻ cells from the parenchyma, choroid plexus, leptomeninges, and perivascular space from SPF and GF mice were fluorescence-activated cell sorting (FACS)-isolated (Fig EV1). Compared to parenchymal microglia, the absolute numbers of CAMs are considerably lower. Therefore, we subjected the isolated myeloid cells to high-throughput single-cell RNA-sequencing (scRNA-seq) using the high-sensitivity method mCEL-Seq2 (Herman et al, 2018; Jordao et al, 2019; Sankowski et al, 2019; Ding et al, 2020) followed by analysis using Seurat version 3 (Stuart et al, 2019) (Fig 1). After quality control, we assembled a transcriptional atlas comprising a total of 2,745 cells recovered from all CNS compartments of SPF and GF animals (Fig 1A). We represented these data using dimensionality reduction with Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP). To discriminate the main cell types in the CNS, we generated maps for the myeloid cell populations based on published signature genes (Jordao et al, 2019) (Fig 1B). Further, the investigated compartment of each cell was displayed in a color-coded UMAP plot (Fig 1C). By using Seurat analysis, we identified 17 molecularly distinct clusters of predominantly innate immune cells from the different CNS compartments (Fig 1D, Table EV1 cell counts per cluster). The majority of cells were attributed as macrophages whereby seven clusters accounted for CAMs (clusters 1, 3, 4, 6, 7, 9, and 13) and four for microglia (clusters 0, 2, 5, and 10). In addition, we detected two clusters for monocytes (clusters 8 and 11).
Figure 1.
and one for DCs (cluster 12), lymphocytes (cluster 16), stromal (cluster 15), and proliferating cells (cluster 14), respectively (Fig 1D). Heatmap representation of top 10 differentially expressed genes (DEG) per cluster highlighted differences across the transcriptional spectrum of CNS macrophages (Fig 1E, Dataset EV1 for cluster marker genes). Most clusters showed a predominant relative expression of CNS macrophages (Fig 1E, Dataset EV1 for clusters). Notably, all DEG were characterized by high expression of microglial core genes, including P2ry12, Tmem119, Hexb, Cystatin 3 (Cst3), and G protein-coupled receptor 34 (Gpr34) (Fig 1D, E, and H). Further, clusters 1, 3, 4, 6, 7, 9, and 13 were attributed to CAMs with elevated expression of typical genes including Mrc1, Cd163, Lyve1, Platelet factor 4 (Pf4), Membrane-spanning 4-domain subfamily A member 7 (Ms4a7), Stabilin-1 (Stab1), and Carbonyl reduc tase 2 (Cbr2). The monocyte cluster 11 was characterized by the expression of Ccr2 and Histocompatibility class II antigen A alpha (H2-Aa) indicating classical Ly6Clo monocytes, while monocyte cluster 8 expressed higher levels of Nuclear receptor subfamily 4 group A member 1 (Nr4a1) indicating non-classical Ly6Che monocytes (Mildner et al, 2017) (Fig 1D, E, and H). Clusters 12 and 16 showed expression of DC and lymphocyte genes, such as Cd209a and Natural killer cell granule protein 7 (Nkg7), respectively (Fig 1D, E, and H). Cluster 15 was composed of a few mixed stromal cells from the brain parenchyma and border regions. Finally, cluster 14 contained cells expressing genes associated with proliferation, including Marker of proliferation mki-67 (Mki67) (Fig 1D, E, and H). Additionally, subsets of cells in cluster 14 showed expression of microglial and CAM signature genes, such as Tmem119 and Mrc1, suggesting a mix of different cell types in this cluster (Fig 1D, E, and H).

To confirm the expression on protein level, we quantified the expression of the typical marker gene CD206 in CAMs (Fig EV2A, B). However, we found no alterations of Iba1+CD206+ CAMs in brains from GF mice compared with SPF controls, suggesting that gut microbiota do not impair the expression of the typical marker gene CD206 in CAMs (Fig EV2A–C).

To analyze gradual changes across clusters, we conducted a pseudotime analysis of microglia and CAMs using StemID2 (Grun et al, 2016). In an unsupervised manner, the algorithm suggested trajectories connecting the microglial (2 – 0 – 10) and CAM (7 – 1 – 4) clusters (Fig EV3) (Herman et al, 2018). The gene expression profiles underlying these trajectories grouped into modules with similar gene expression patterns, and their z-scores were visualized for each of the two compartments of interest (Fig EV3, Datasets EV3 and EV4 for genes along the microglial and CAM trajectories). Notably, clusters that were enriched for cells from GF mice (i.e., cluster 10 for microglia) showed higher expression levels of Apoe while clusters enriched for cells from SPF mice showed higher levels of genes associated with immune function, including Lymphocyte antigen 86 (Ly86, also known as Mld-1) (Fig EV3, Datasets EV3 and EV4). These findings suggest that the presence of microbiota drives microglia and CAM polarization toward an immunogenic transcriptional phenotype.

To investigate the functional states of the identified macrophage clusters, we performed gene ontology (GO) enrichment analysis based on cluster-enriched gene sets (Appendix Fig S1, Dataset EV5 for an overview of enriched GO terms). Most microglial and CAM clusters showed enrichment for terms linked to immune function such as Chemotaxis, Phagocytosis, and Myeloid cell homeostasis (Appendix Fig S1). Further, several myeloid cell clusters were characterized by the enrichment of the GO terms Regulation of response to external stimulus and Response to bacterium. The term Antigen processing and presentation of peptide antigen was associated with all CAM clusters except cluster 3 that was mainly assembled by cells from GF brains. Notably, the microglial clusters 0 and 10 that were mainly composed of GF-derived cells showed either no association with this term (cluster 0) or only 5 associated genes (cluster 10) (Appendix Fig S1, Dataset EV5). Furthermore, the term Cytokine secretion was not correlated with the two clusters 0 and 10 possibly suggesting attenuated immune priming. Clusters 7, 1, and 13 (attributed to CAMs) showed enrichment of the GO term Interleukin-1 production (Appendix Fig S1). The proliferating cell cluster showed enrichment of the term Regulation of mitotic cell cycle and Interferon gamma production was solely attributed to lymphocytes (cluster 16) (Appendix Fig S1).

These findings suggest that CAMs and microglia are affected by the microbiota composition.

**Microbiota-dependent transcriptional heterogeneity of microglia**

To further characterize CNS myeloid cells from SPF- and GF-housed mice, we first analyzed their morphology (Appendix Fig S2). As previously shown (Erny et al, 2015), we observed enlarged microglial cells with significantly increased surface area and volume in the cortex of GF mice compared with SPF-housed mice (Appendix Fig S2A). In contrast, cpMΦ, mmMΦ, and pvMΦ exhibited no significant morphological divergences at the respective border regions (Appendix Fig S2B–D).

By using bulk RNA-seq, we determined previously that microglia from GF mice display an altered gene expression signature as compared to microglia from SPF mice (Erny et al, 2015). However, it remained unclear whether the composition and heterogeneity of microglia on a single-cell level is changed. Therefore, we assessed potential compartment-specific signatures of microglia from SPF and GF mice in the subset of CNS parenchyma-derived cells (Fig 2). This revealed 828 individual microglial cells derived from SPF and GF brains with typical marker expression which clustered in one scattered microglia cloud with eight subsets, suggesting a spectrum of gradual transcriptional changes (Fig 2A–C, Table EV2 for cell counts per cluster and condition and Dataset EV6 for cluster marker genes). Microglia from clusters 3, 4, and 7 were predominantly GF-derived, whereas clusters 2 and 6 comprised significantly more SPF-derived cells (Fig 2D, Dataset EV7 for hypergeometric test statistics). Analysis of SPF and GF microglia showed several DEG including marked higher abundance of Ly86, a marker linked to cell activation (Jordao et al, 2019) and Cd180 which forms a cell surface receptor complex with Ly86 (Nagai et al, 2002) for SPF microglia. Comomitant GF microglia featured higher expression of Complement C1q B chain (C1qb) and DNA-damage-inducible transcript 4 (Ddit4).
Figure 2.
In sum, these findings showed that the overall composition and heterogeneity of microglia harbors specific microbiota-dependent alterations while microglial density is highly plastic and can be modulated by complex microbiota and is mediated by the microbiota-derived metabolite SCFAs.

**Complex microbiota controls choroid plexus macrophage properties**

Next, we profiled the CAM populations from the choroid plexus separately, whereby 487 single cells from healthy SPF and GF mice resulted in eight subsets with statistically significant enrichment of SPF-derived cells in cluster 5, while cluster 1 was predominantly composed of GF-derived cells (Fig 3A–D, Table EV3 for cluster cell counts per condition, Dataset EV9 for cluster marker genes and Dataset EV10 for hypergeometric test statistics). We observed two transcriptionally distinct CAM subsets corresponding to cpMΦ (clusters 0, 1, and 5) and Kolmer epiplexus (CPep) cells (clusters 2–4) (Fig 3C). While cpMΦ expressed typical CAM signature genes (Fig 1A left), CPep cells displayed expression of genes usually attributed as typical microglial markers (Van Hove et al., 2019) (Fig 1A right). Differential gene expression analysis between SPF and GF cpMΦ showed higher expression of Ly66 in SPF and Apoe in GF cpMΦ, respectively (Fig 3E, Dataset EV11 for overview of all DEGs for this comparison). Additionally, DEG of SPF CPep cells included Cds2 and Guanine nucleotide binding protein, alpha stimulating (Gnas) while GF CPep cells showed higher expression of Apoe and Abca1, suggesting that GF cpMΦ and CPep cells might mirror a rather immature state analogous to GF microglia (Figs 3E and EV5A). Overall, Cst3, Sparc, P2ry12, and Hexb showed higher abundance in clusters 2–4 attributed to CPep cells, Apoe, Mrc1, and Stab1 in clusters 0 and 1 referring to cpMΦ (Fig 3F and G, Dataset EV9). P2RY12 positively labeled iba1+ CAMs were identifiable in the choroid plexus by immunohistochemistry, whereby we found no differences due to the GF status (Fig EV2D–F). In addition, we noticed significantly more APOE/iba1+ CAMs in the choroid plexus of GF mice compared with SPF controls supporting the scRNA-seq data (Fig EV2G–I). Besides, the dataset contained some proliferating (cluster 7) and stromal cells (cluster 6) and showed expression of,
Figure 3.
e.g., *Purkinje cell protein 4-like 1* (*Pcp4l1*), *Clusterin* (*Clu*) and *Mki67*, respectively (Fig 3F–G, Dataset EV9).

Next, we quantified Iba1+ CAMs in the choroid plexuses of lateral and fourth ventricles of SPF and GF animals and found a marked reduction of stromal cpMΦ in GF mice (Fig 3H and I), which is in contrast to increased numbers of parenchymal microglia in these animals (Fig EV4A and B). Remarkably, the density of CP<sub>epi</sub> was not affected by gut bacteria (Fig EV5B and C). Further, upon treatment with ABX, the number of stromal cpMΦ significantly declined (Fig 3H and J), which is again in contrast to stable microglial numbers (Fig EV4A and C) indicating that CNS myeloid cells are divergently regulated by gut microbiota. While a highly limited composition of gut bacteria—as reflected in both ASF and sDMDMm2 mice—caused a significant reduction of stromal cpMΦ numbers (Fig 3H, K, and L), mice recolonized with complex gut microbiota from SPF donors showed restored stromal cpMΦ densities as observed in SPF animals (Fig 3H and K). Next, we investigated whether distinct MAMPs influence stromal cpMΦ densities. However, TLR2-, TLR3-, TLR4-, TLR7-, and TLR9-deficient mice did not manifest altered stromal cpMΦ numbers (Fig 3H and M). Surprisingly, treatment with SCFAs failed to restore stromal cpMΦ quantities (Fig 3H and N), which contrasts with their counterparts in the CNS parenchyma, the microglia (Fig EV4A and G). Additionally, FFAR2 deficiency did not affect stromal cpMΦ density (Fig 3H and O), indicating a dispensable role of SCFAs/FFAR2 signaling for stromal cpMΦ.

Overall, these findings support the presence of distinct CAM subpopulations in the choroid plexus that are divergently shaped by microbiota with a high diversity while the signaling from the gut to the choroid plexus seemed to be independent of SCFA.

**Single-cell analysis of perivascular macrophages in GF mice**

Subsequently, we investigated 885 cells derived from the perivascular space from SPF- and GF-housed mice (Fig 4A and B, Table EV4 for cluster cell counts per condition). We observed five subsets (clusters 0, 1, 2, 6, and 9) of pvMΦ with enrichment of SPF cells in cluster 2 and GF cells in cluster 6 (Fig 4C and D, Dataset EV12 for cluster marker genes and Dataset EV13 for hypergeometric test statistics). Furthermore, few microglia, cDCs, lymphocytes, and Ly6<sup>hi</sup> and Ly6<sup>lo</sup> monocytes were captured (Fig 4C). DEG analysis of pvMΦ from SPF and GF animals showed higher expression levels of Ly86 and Fc receptor-like S, scavenger receptor (Frs) in SPF cells and Cd163, Lyve1, and Cd209b in GF pvMΦ (Fig 4E, Appendix Fig S4, Dataset EV14 for overview of all DEGs for this comparison). Higher Apoe mRNA levels were detected in cluster 6 that was mainly composed GF-derived cells (Dataset EV12). Increased proportion of APOE<sup>+</sup>Iba1<sup>+</sup> cells in the perivascular space was confirmed by immunohistochemistry (Fig EV2G–I). pvMΦ expressed typical markers for CAMs such as *Mrc1*, *Cd163*, *P4H*, *Stab1*, and *Cbr2* (Fig 4F and G), whereas *Ctsc* gene transcripts presented heterogeneous expression patterns with higher abundance of *Ctsc* transcripts in cluster 0 (Fig 4F, Dataset EV14). Clusters 3 and 4 were mainly composed of Ly6C<sup>lo</sup> and Ly6C<sup>hi</sup> monocytes, respectively, and showed relatively increased transcripts for *Ccr2* or *Nrg4a1* (Fig 4C, F, and G). Microglia (cluster 7) presented higher *Hexb* expression and lymphocytes (cluster 8) were characterized by higher numbers of *Nkg7* mRNA transcripts (Fig 4C, F, and G).

Furthermore, we explored pvMΦ in the perivascular space of cortical areas by Iba1 immunohistochemistry in SPF and GF mice (Fig 4H and I). However, we discerned no shifts for pvMΦ numbers in mice lacking microorganisms, as well as by microbiota eradication through ABX treatment, in mice with limited gut bacteria composition, after recolonization with diverse composed microbiota, SCFAs application, and in mice lacking TLR2, TLR3, TLR4, TLR7, TLR9, or FFAR2 deficiency (Fig 4I–O), indicating that pvMΦ quantities are not affected through constitutive (GF), induced (ABX) gut microbiota depletion or limited complexity (ASF, sDMDMm2) at steady-state conditions.

In sum, these findings suggest that the transcriptional profile of pvMΦ is controlled by intestinal microbiota whereas cell numbers remain stable.

**The transcriptional profile of meningeal macrophages under GF conditions**

Next, we analyzed the transcriptional profiles of 545 leptomeningeal cells from mice housed under SPF and GF conditions, respectively.
Figure 4.
We found three subsets of mMMφ (clusters 0, 2, and 7) with significantly increased numbers of SPF-derived macrophages in clusters 0 and 2 (Fig 5A–D, Table EV5 for cluster cell counts per condition, Dataset EV15 for cluster marker genes and Dataset EV16 for hypergeometric test statistics). Besides mMMφ, microglia (clusters 1, 3, 5, 6, and 8), Ly6c(+) (cluster 9) and Ly6c(−) monocytes, and cDCs (both in cluster 4) were captured (Fig 5C). In contrast to cpMMφ, pvMMφ, and microglia, DEG analysis showed no major differences between SPF and GF mMMφ except higher expression of *Chondroitin sulfate proteoglycan 5* (*Cspg5*) in GF mMMφ (Fig 5E). Overall, mMMφ expressed *Mrcl*, *C1d63*, *Pf4*, *Stab1*, and *Cbr2* (Fig 5F and G). The percentage of APOE*+/Iba1* meningeal cells was not affected by the GF status (Fig EV2C–I). N4a1 was mainly expressed in Ly6c(+) monocytes, Ccr2 in Ly6c(+) monocytes and alongside with Cld209r in cDCs (Fig 5F and G). *Hexb* and *P2ry12* were mainly expressed by microglia with a reduced expression of the latter gene in clusters 6 and 8 that were mainly composed by GF-derived microglia (Fig 5F and G).

To determine whether the gut microbiota is essential for the mMMφ numbers, we examined Iba1+ mMMφ in the leptomeninges by immunohistochemistry (Fig 5H). Overall, we noticed no alterations for Iba1+ mMMφ in mice lacking microorganisms or by different microbiota manipulation strategies, namely through recolonization, SCFA supplementation, TLR2, TLR3, TLR4, TLR7, TLR9, and FFAR2 deficiency (Fig 5H–O), indicating that gut microbiota does not affect mMMφ numbers.

Collectively, these results suggested minor influences of gut bacteria on mMMφ.

**Microbiota-dependent innate immune response of CNS myeloid subsets to acute cerebral LCMV infection**

Macrophages are the first responders to infections of the brain, mediating early pathogen control and initiating downstream immune reactions (Prinz et al., 2017; Kierdorf et al., 2019). To examine the functional consequences of CNS macrophage alterations in...
Figure 5.
mice lacking microbes, we infected the animals with LCMV intracerebrally (i.c.) and analyzed CNS myeloid cell expansion after 4 days (Fig 6A and B). Microglia from SPF mice reacted with a strong increase of cell density, whereas the virus-induced microgliosis was less prominent in GF mice. A similar response with elevated Iba1+ cells numbers was present in the choroid plexus, the leptomeninges, and the perivascular space from mice with gut microbiota while mice devoid of microbiorganisms presented diminished cell expansion (Fig 6A and B).

Accordingly, the number of proliferating Iba1+Ki67+ microglia and to a lesser degree CAMs was increased under SPF conditions (Fig 6C). To gain further insights into the activation state of the CAMs in LCMV infected brains, we quantified the proportion of CD74+Iba1+ CNS macrophages (Fig 6D). Recently, CD74 was reported as a marker for activated CAMs (Jordao et al., 2019). Notably, we found increased CD74+Iba1+ microglial and CAM numbers in all compartments in SPF-housed mice challenged with LCMV compared to PBS controls (Fig 6D). In contrast, in LCMV challenged GF mice the numbers of CD74+Iba1+ remained largely unaffected.

In sum, these data suggest that in GF mice that are devoid of intestinal microorganisms, the innate immune response of CAMs against an acute challenge by an RNA virus is impaired.

Perivascular macrophages in AD transgenic mice clear Aβ in a microbiota-dependent manner

To gain further insights into the functional consequences of microbiota-dependent properties of CAMs, we assessed the role of CAMs in a more chronic disease state. To this end, we took advantage of the 5xFAD mouse model of Alzheimer’s disease (AD) and observed Aβ depositions in the leptomeninges of the cortex, choroid plexus, hippocampal brain parenchyma, and perivascular space of 4-month-old male SPF, GF, and ABX-treated 5xFAD mice (Fig 7A–C). Recently, we determined that the extent of the Aβ burden in hippocampal regions is gut microbiota-dependent (Mezo et al., 2020). Accordingly, we observed reduced Aβ depositions in the hippocampi of the GF and ABX-treated 5xFAD mice (Fig 7C and D). Notably, we found significantly increased cell densities of mMΦ, cpMΦ, and pvMΦ in the respective anatomical regions of SPF 5xFAD mice compared with GF and ABX-treated 5xFAD mice and matched wild-type (WT) controls (Fig 7E–G). To explore the functional role of CAMs in Aβ pathology, we determined the percentage of CAMs that incorporated Aβ by double immunohistochemistry for Iba1 and Aβ (Fig 7A–C). In contrast to mMΦ and cpMΦ, Aβ-containing Iba1+ pvMΦ was significantly more frequent in transgenic 5xFAD mice housed under GF conditions compared with SPF 5xFAD mice (Fig 7H–J). Remarkably, the percentage of Iba1+ Aβ+ pvMΦ was also significantly increased after ABX treatment of 5xFAD mice (Fig 7J) suggesting a high plasticity of this effect. We verified this finding by performing ex vivo flow cytometric-based analysis of Aβ uptake using metoxy-X-O4 staining (Mezo et al., 2020) and detected significantly increased percentage of metoxy-X-O4+ pvMΦ under both GF conditions and upon ABX treatment (Fig 7K).

In sum, our data suggest that host microbiota regulate Aβ uptake only in pvMΦ in AD transgenic mice and thus may contribute to disease outcome by impaired Aβ clearance.

Discussion

The present study provides a comprehensive analysis of the microbiota-dependent transcriptional landscapes of CNS-resident myeloid subsets. We determined steady-state cell-specific profiles of CNS myeloid cells throughout the various analyzed CNS regions. Previously, we have shown that proper maturation of microglia critically depends on immunological priming by soluble factors produced by a complex microbiota (Enny et al., 2015). However, the contribution of gut microbiota on other myeloid cells at CNS interfaces remained unclear. Due to their prominent position at the CNS border regions, CAMs are important immunological gatekeepers of the CNS (Prinz et al., 2017; Jordao et al., 2019; Kierdorf et al., 2019; Brioschi et al., 2020). Particularly, the CP has been identified as crucial for the immunological milieu of the brain parenchyma and cerebrospinal fluid with implications for brain development and aging (Baruch et al., 2014; Cui et al., 2020; Shipley et al., 2020).

For microglia, cpMΦ and pvMΦ, but not mMΦ we observed increased expression of Apoe in GF mice which was further verified on protein level. Increased Apoe expression might be indicative for a rather immature state of the respective macrophage populations. At least for microglia, it is known that Apoe is being downregulated during embryonic development (Butovsy et al., 2014). Referred to our previous bulk RNA-seq analysis, we detected significantly less DEG in SPF and GF microglia possibly due to the reduced detection/sensitivity of lowly expressed genes in scRNA-seq (Kharchenko et al., 2014). As for the cpMΦ and pvMΦ, we observed a wider spectrum of transcriptionally distinct clusters compared to mMΦ, which were largely unaltered. All three CAM subsets featured clusters with elevated expression of typical CAM markers such as Mrcl, Stab1.
Figure 6.
and Cbr2. SPF cpMΦ and pvMΦ exhibited increased expression of Ly86 as microglia did as a sign for cell activation (Jordao et al., 2019). In contrast, CAMs of the choroid plexus additionally had clusters with P2ry12-expressing cells. These latter cells likely represent Kolmer epiplexus cells that are transcriptionally distinct from stromal cpMΦ (Jordao et al., 2019; Van Hove et al., 2019). Both cell populations in the choroid plexus featured microbiota-dependent transcriptional alterations including upregulated Ly86 and downregulated Apoe expression in SPF stromal cpMΦ and CPepi compared with GF conditions. Further, only stromal cpMΦ numbers but not Iba1

![Image](https://example.com/image1.png)

**Figure 7.**

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CPριη numbers were highly sensitive to different microbiota manipulation strategies which is in line with a previous report, highlighting the critical responsiveness of cpMF to the microbiota (Van Hove et al., 2019). In contrast to microglia that presented increased numbers in GF animals (Erny et al., 2015), the complete absence of gut commensals in GF-housed mice as well as postnatal depletion of gut bacteria with ABX and a limited gut microbiota composition caused a reduction of stromal cpMF. Conversely, recolonization with complex gut microbiota normalized stromal cpMF numbers indicating that stromal cpMF requires a continuous input from the resident gut bacteria. Gut microbiota-derived MAMPs were shown to mediate steady-state myelopoiesis in the spleen and bone marrow (Khosravi et al., 2014). However, we did not observe any alterations in stromal cpMF numbers by analyzing mice with TLR2, TLR3, TLR4, TLR7, and TLR9 deficiency, suggesting other mechanisms than activation of these patter recognition receptors (PRR) for microbiota-driven effects on stromal cpMF. In contrast to microglia, both SCFAs and the SCFA receptor FFAR2 were redundant for stromal cpMF density under homeostatic conditions, probably due to the dual origin of stromal cpMF (Goldmann et al., 2016; Van Hove et al., 2019). In contrast to most peripheral tissue macrophages, CAMs and microglia originate from early erythromyeloid yolk sac-derived progenitors during embryonic development (Goldmann et al., 2016; Utz et al., 2020). It was demonstrated that stromal cpMF is the only CAMs that are replaced by bone marrow-derived cells over time and do not fully self-renew as CPριη and their relatives in the meninges, perivascular space, and brain parenchyma (Goldmann et al., 2016). Therefore, we speculate that reduced stromal cpMF numbers might be caused by reduced numbers of bone marrow-derived monocytes as it was described in GF mice highlighting potential ontogeny-related cellular differences in CNS macrophages (Khosravi et al., 2014; Zhang et al., 2015). Interestingly, it was further suggested that diminished monocyte numbers in the bone marrow of GF animals might be caused by decreased levels of growth factors such as CSF1 (Khosravi et al., 2014; Zhang et al., 2015). Further, reduced monocyte numbers in GF mice could not be normalized through the administration of SCFAs whereby the same SCFA treatment regime was applied as in our analysis (Khosravi et al., 2014). Alternatively, it might be possible that levels of local growth factors are altered in the choroid plexus of GF mice and thereby causing decreased numbers of stromal cpMF independent of monocyte-derived cells. Remarkably, mMF and pvMF did not display any changes in their cell densities in GF animals and upon microbiota manipulation with ABX, ASF, sDMDDm2, or SCFA treatments. We speculate that this might be caused through overall relatively low cell numbers of mMF and pvMF in the respective compartments.

Our results further support the idea that a continuous input from complex microbiota is necessary for proper CNS myeloid cell function. Upon acute challenge with LCMV, expansion of CAMs and microglia, as well as the expression of the marker CD74 that is associated with activated CAMs (Jordao et al., 2019), was compromised in CAMs of GF mice, indicating that the innate immune response of CNS macrophages is collectively attenuated in the absence of intestinal microbes.

In order to gain further functional insights, we characterized the Aβ-associated pathology using the transgenic 5xFAD mouse model for AD. 5xFAD mice feature Aβ debris around blood vessels, a situation known as cerebral amyloid angiopathy (CAA) (Giannoni et al., 2016). Cerebral Aβ levels can be regulated by different Aβ clearance mechanisms, e.g., via the blood–brain barrier (BBB) (Heneka et al., 2015), and we therefore asked whether microbiota may influence Aβ clearance by CAMs (Mildner et al., 2011). GF 5xFAD mice displayed reduced Aβ pathology, whereby our data suggested for the first time elevated Aβ clearance by pvMF but not mMF or cpMF in GF 5xFAD mice. Previous results indicated ameliorated Aβ levels in GF 5xFAD mice caused by enhanced microglial Aβ uptake (Mezo et al., 2020), while Aβ clearance by pvMF might further facilitate removal of Aβ. Notably, the Aβ uptake of microglia was not inducible upon ABX treatment although the Aβ pathology was significantly diminished in these mice (Mezo et al., 2020). We speculate that this might be primarily due to augmented Aβ clearance by pvMF upon ABX treatment. Therefore, compromised perivascular clearance of Aβ might be a crucial factor in the accumulation of Aβ in CAA and may be a suitable target for new therapeutic approaches (Hawkes et al., 2014). Interestingly, several genes (including Apopo and P2ry12) that have been recently linked to Aβ-activated microglia in AD mouse models (Keren-Shaul et al., 2017; Krasmann et al., 2017) displayed elevated expression in microglia and CAMs from GF mice under steady-state conditions, suggesting a possible microbiota-dependent influence on this disease-associated activation state of CNS macrophages. In our previous study, we ruled out the...
notably, the increased Aβ uptake by microglia and pvMF in GF and ABX-treated 5xFAD mice resulted in preserved neuronal density in the hippocampus and related cognitive function (Mezo et al., 2020).

In summary, we found that host microbiota is crucial for the properties of selective CNS macrophages at border regions during homeostasis and activation upon acute viral challenge and chronic neurodegeneration. We revealed distinct microbiota-dependent cellular Aβ-clearance mechanisms in the 5xFAD mouse model of AD. These results may further widen our understanding and treatment of CNS macrophage-mediated inflammatory and neurodegenerative diseases of the CNS.

Materials and Methods

Mice

Conventional SPF mice on C57BL/6J background were purchased from Janvier labs (Saint-Berthevin, France). Germ-free C57BL/6J mice were obtained from the Clean Mouse Facility (Bern, Switzerland). All mice (mixed sex) were used at 6–10 weeks of age. ASF and recolonized ASF mice on a C57BL/6J (females) were provided by Bärbel Stecher (Max-von-Pettenkofer Institute, LMU Munich, German Center for Infection Research) (Stecher et al., 2010). ASF mice colonized with Bacteroides distasonis (strain ASF 519), Lactobacillus salarius (strain ASF 361), and Clostridium cluster XIV (ASF 356) were housed under germ-free conditions in a flexible film isolator with HEPA-filtered air and autoclaved chow and bedding. For recolonization, ASF mice were co-housed with conventional SPF donor mice in the same cage for six weeks. Mice with a stable defined moderately diverse mouse microbiota (sDMDDmm2) (Uchimura et al., 2016), also known as Oligo-MM12 (Garzetti et al., 2017), were obtained from Andrew Macpherson and Mercedes Gomez de Agüero (DBMR, Bern, Switzerland). For substantial depletion of the microbiota, SPF mice were provided with drinking water containing 1 mg/ml cefoxitin (Santa Cruz Biotechnology, Heidelberg, Germany), 1 mg/ml gentamicin (Sigma-Aldrich, Taufkirchen, Germany), and 1 mg/ml vancomycin (Hexal, Holzkirchen, Germany). All mice (mixed sex) were used at 6 months of age) on a C57BL/6J background co-expressing human APPK670N/M671L (Sw)-β16 (Fl) and PS1M146L-L286V under the control of the neuron-specific Thy-1 promoter (Oakley et al., 2006). Mice were housed under specific pathogen-free (SPF) conditions under a 12-h light, 12-h dark cycle with food and water ad libitum at the Center for Experimental Models and Transgenic Service (CEMT, Freiburg, Germany). GF 5xFAD mice were generated via embryo transfer by Kathleen McCoy. GF heterozygous 5xFAD and WT littermates were obtained from the Clean Mouse Facility (Bern, Switzerland). In order to deplete microbiota, 5xFAD mice and littermate controls were treated orally via drinking water with a mixture of antibiotics (ABX), containing 1 mg/ml vancomycin (Hexal), 1 mg/ml cefoxitin (Santa Cruz Biotechnology), 1 mg/ml gentamicin (Sigma-Aldrich), and 1 mg/ml metronidazole (Sigma-Aldrich) for 2 months and analyzed an age of 4 months. All animal experiments were approved by the Ministry for Nature, Environment and Consumers’ Protection of the State of Baden-Württemberg and were performed in accordance with the respective national, federal, and institutional regulations (permit numbers G19-02 and X16-04A).

Histology

Histology was performed as described recently (Erny et al., 2015). Brains were removed and fixed in 4% phosphate-buffered formalin. Brain tissue was dissected, and parasagittal sections were embedded in paraffin before staining with Iba1 (Wako, Osaka, Japan) for microglia and CAMs. At least 3–4 parasagittal brain sections per mouse were evaluated using Bresser MikroCamLabII (Bresser, Rheine, Germany).

For immunofluorescence staining, coronal cryosections with a thickness of 25 µm from adult brain tissue were prepared as described previously (Mezo et al., 2020) and incubated free-floating first with anti-Iba1 antibody (Wako, 019-19741, Osaka, Japan), for 24 h (dilution 1:500 at 4°C). Subsequently, Alexa Fluor 488-conjugated secondary antibody (Life technologies, Darmstadt, Germany) was added at a dilution of 1:500 for 24 h at 4°C. Compact Aβ-plaques, as well as 1-16 Aβ precursor forms, were visualized by using anti-6E10 antibody (1:1,000, 803004, BioLegend, San Diego, USA) at 4°C for 24 h, followed by incubation with corresponding conjugated secondary antibodies Alexa Fluor 568 (1:500, Life technologies) for 24 h at 4°C. DAPI was used as a nuclear stain for 10 min at RT. Aβ-plaque load and density of Iba1-immunoreactive pvMF were quantified throughout the entire hippocampus. Further cortical mMF and cpMF were assessed. Images were taken with a BZ-9000 Biorevo microscope (Keyence, Neu-Isenburg, Germany), and the number of Iba1-positive cells was determined using BZ-II Analyzer (Keyence, Neu-Isenburg, Germany).

Paraffin-embedded brains were cut sagittally into 3 µm thin sections, and immunofluorescence labeling was performed as described previously with slight modifications (Mezo et al., 2020) by using following primary antibodies: anti-CD206 antibody (1:1,000, 803004, BioLegend), anti-Iba1 (1:500, NB100-1028, Novus Biologicals, Wiesbaden, Germany or Wako, 019-19741, Osaka, Japan), anti-CD74 (1:200, P452215, Thermo Fisher, Waltham, USA), anti-CD163 (1:200, A5-55043A, AnaSpec, Fremont, USA), anti-Ki67 (1:200, ab15580, Abcam, Cambridge, UK), and anti-APoE (1:50, A9474, Merck, Darmstadt, Germany), upon antigen retrieval (pH 6.0 citrate) at 4°C for 24 h. Subsequently,
30 μm parasagittal cryosections from adult brain tissue were stained with anti-Iba1 (1:500, 019-19741, Wako, Osaka, Japan), CD31 (1:200, 550274, BD Pharmingen, Heidelberg, Germany), and ER-TR7 (1:1,000, ab51824, Abcam, Cambridge, UK) for 48 hours (dilution 1:500 at 4°C), followed by Alexa Fluor 488- and 568-conjugated secondary antibody (Life technologies, Darmstadt, Germany) staining, which was added at a dilution of 1:500 overnight at 4°C, as described previously (Erny et al., 2015). Nuclei were counterstained with DAPI. Imaging was performed on an Olympus Fluoview 1000 confocal laser scanning microscope (Olympus, Hamburg, Germany) using a 20 × 0.95 NA objective. Z stacks were done with 1.14-μm steps in z direction, and 1,024 × 1,024 pixel resolution was recorded and analyzed using IMARIS software (Bitplane, Zurich, Switzerland). At least three cortical cells were reconstructed per mouse.

Myeloid cell isolation

To enrich the yield of CAMs, we pooled 20 mice per group. The different CNS compartments were separated, and myeloid cells were isolated as described before with slight modifications (Erny et al., 2015; Jordao et al., 2019). In short, the leptomeninges were dissected from the brain to obtain separate samples of the leptomeninges and of the parenchyma and perivascular space. All choroid plexuses were removed from the ventricles of the brain. Myeloid cells from the parenchyma and perivascular space were isolated using a 37% Percoll gradient (Sigma-Aldrich) at 800 g (4°C) from the homogenized tissue. Tissue from the choroid plexuses and leptomeninges was treated independently by mechanical dissociation through a 70-μm cell strainer to obtain cell suspensions. Dead cells were stained using the Fixable Viability Dye eFluor 780 (1:1,000, eBioscience, San Diego, USA) followed by incubation with Fc receptor blocking antibody CD16/CD32 (BD Bioscience, Heidelberg, Germany). Afterward, cells were stained with primary antibodies directed against CD11b (M1/70), CD45 (50-F11; eBioscience, San Diego, USA), CD206 (C068C2), B220 (RA3-6B2; BioLegend), CD5 (ASP34-2), Ly6C (AL-21), and Ly6G (1A8; BD Bioscience, Heidelberg, Germany) with a dilution of 1:200 at 4°C for 30 min. Viable single cells from n ≥ 5 animals per condition were pooled and sorted with a MoFlo Astrios (Beckman Coulter, Krefeld, Germany) into 384-well plates and further processed.

Single-cell RNA-seq analysis

Samples were processed using the mCel-Seq2 protocol (Herman et al., 2018). 28 libraries were sequenced in batches of eight libraries with 192 cells each per lane on an Illumina HiSeq 3000 sequencing system (pair-end multiplexing run) at a depth of ~130,000–200,000 reads per cell. Features with a correlation coefficient of > 0.65 with low-quality cell marker Kcnq1ot1 were excluded from analysis (Grun et al., 2016). 2,815 cells containing more than 500 features (cell numbers per condition—SPF: 1,142; GF: 1,673; cell numbers per compartment—microglia: 830, cpMΦ: 489, pvMΦ: 942, mMΦ: 554) were analyzed using Seurat version 3 (Stuart et al., 2019). Doublet detection was performed with an estimated multiplet rate of 2.5% on a Seurat object with all cells using the DoubletFinder R package version 2.0.3 leading to a final dataset of 2,745 cells. A total of 19,488 gene loci were quantified with a median of 1,489 features per cell ± 735.44 (S.D.). Data analysis was performed using the Seurat version 3 algorithm (Herman et al., 2018) using the SCTransform workflow with default parameters on the first 15 principal components of the data. The resolution parameter for the FindClusters function was chosen at 1.2 after visual inspection of the resulting clustering tree using the clusterTree R package version 0.4.3 (https://github.com/lazappi/clustree). Cluster markers for heatmap visualizations were obtained using the FindMarkers Seurat function. Direct comparisons of microglia and CAMs from GF and SPF mice were conducted using the FindMarkers Seurat function.

Cluster enrichment analysis

Enrichment analysis for GF or SPF-derived cells in a cluster was done using a hypergeometric test implemented in R under the phyper function. This test considers the number of cells from a condition in per cluster with respect to all cells from this condition in the dataset, all cells from the second condition in the dataset and the number of cells in each cluster. The p-value returns the probability that number n or more cells from a condition could be found in a given cluster by chance. Probabilities of < 0.05 were considered statistically significant. Multiple testing correction was achieved using the Benjamini–Hochberg method.

Gene ontology analysis

Enrichment of gene ontology (GO) term analysis was analyzed using the clusterProfiler R package version 4.0.5 (Yu et al., 2012). Differentially expressed cluster marker genes were used as input gene lists. All quantified genes in the dataset are used as background genes.

Pseudotime analysis of single-cell transcriptomes

The StemID2 (contained within RaceID version 0.2.3) and FateID version 0.2.0 packages were used for pseudotime trajectory analysis of the single-cell RNA-seq with default settings (Grun et al., 2016; Herman et al., 2018). After construction of a lineage tree using the nearest neighbor mode (nmode=TRUE) with default parameters of StemID2, a list of significant links was chosen for each cell type in question. A filtered gene expression matrix was used as input for the pseudotime gene expression analysis of cells along the list of selected links. Lowly expressed genes were removed with the filterset FateID function. The getsom and procsom FateID functions grouped genes with similar gene expression profiles into modules on a self-organizing map. Smoothed z-scores of the genes in these modules were visualized.

LCMV challenge

Lymphocytic choriomeningitis virus strain WE was propagated and titrated as plaque-forming units (PFU) on L929 cells as described
before (Herz et al., 2009). PFU were multiplied by the factor 10 to be converted into infectious units (IU). Mice were infected by intracerebral inoculation of 10^3 IU into the right hemisphere.

**Ex vivo Aβ phagocytosis assay**

Mice were injected intraperitoneally with methoxy-X-O4 (Tocris) (10 mg/kg bodyweight), a fluorescent congo red derivate, in a DMSO/PBS mixture as described previously (Mezo et al., 2020). After 3 h, hippocampi were collected and pvMΦ were assessed as CD11b+CD45^hi cells. Percentage of methoxy-XO-4-positive pvMΦ were determined by flow cytometry using a FACS Canto II (BD Bioscience) and analyzed with FlowJo software (Tree Star).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism (GraphPad Software, version 5.0, La Jolla, USA). Tabular data were processed using the tidyverse suite of packages (Wickham & Wickham, 2017) and visualized in the R programming environment version 4.1.0 (R Core Team, 2013) using the ggplot2 package version 3.3.5 (Wickham, 2016).

All data were tested for normality applying the Shapiro–Wilk normality test. If normality was given, an unpaired t-test, one-way ANOVA followed by Tukey’s post hoc comparison test or two-way ANOVA followed by Tukey’s post hoc comparison test was applied, respectively. Differences were considered significant when P-value < 0.05. To obtain unbiased data, experimental mice were all processed together, and cell quantifications were performed blinded by two scientists independently and separately.

**Data and software availability**

Raw and processed single cell RNA-seq data including the metadata and Seurat objects are available at Gene Expression Omnibus (GEO: GSE178956). The code book for the present study is available under: https://github.com/rsankowski/sankowski_et_al_murine_CAMs.

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