Microglia promote anti-tumor immunity and suppress breast cancer brain metastasis

Editor summary:

Evans, Blake, Longworth and colleagues identify and characterize a tumour-suppressive role for microglia which mediate a pro-inflammatory response to restrict brain metastasis in breast cancer.

Reviewer Recognition:

Nature Cell Biology thanks the anonymous reviewers for their contribution to the peer review of this work.

Figure or Table # Please group Extended Data items by type, in sequential order. Total number of items (Figs. + Tables) must not exceed 10.	Figure/Table title One sentence only	Filename Whole original file name including extension. i.e.: Smith_ED_Fig1.jpg	Figure/Table Legend If you are citing a reference for the first time in these legends, please include all new references in the main text Methods References section, and carry on the numbering from the main References section of the paper. If your paper does not have a Methods section, include all new references at the end of the main Reference list.
Extended Data Fig. 1	Analysis of TAMs and astrocytes in BCBM	ED_Fig1.pdf	a. Whole mount brightfield and fluorescent microscopy images of metastatic brains (Met1-3) used to generate the scRNA-seq dataset described in **Figure 1d**. Metastatic lesions are GFP^+ (green). Results are representative of a single experiment. Scale bar = 50 mm. b. IF staining shows $IBA1^+$ cells (red) in normal human brain and three resected patient BCBM tumors. Insets show cell morphology, exhibiting evenly spaced, ramified microglia in normal human brain contrasting heavily infiltrated ameboid microglia in BCBM patients.

			Results are representative of a single experiment. Scale bar = 50 μm. c. Representative FACS plots show gating for single, live (Sytox negative) myeloid cells ($CD45^+CD11b^+$), astrocytes ($CD45^-ASCA2^+$) and 231BR cells ($CD45^-GFP^+$) isolated for scRNA-seq. d. Identification of mouse and human cells by the frequency of reads that align to the mm10 mouse genome. Cutoffs used to identify mouse cells (>0.875 aligned, n=51,418 cells), human cells (<0.05 aligned, n=7336 cells) and doublets (0.05-0.875 aligned, n=913 cells) are shown. e. Violin plots show cell distributions for key quality control metrics pre- and post- filtering and removal of poor quality cells. Cells were removed that displayed <500 or >2000 genes (nFeature_RNA), or >10% of genes mapped to the mitochondrial genome (percent mito genes).

			f. Bar chart shows the frequency of cells contributed by each mouse that localize to each cell type in **Figure 1f**. g. tSNE plot shows astrocytes colored by control or metastatic condition. h. Volcano plot shows genes differentially expressed (n=6,542) between astrocytes from control and metastatic brains determined by Wilcoxon rank sum test, ($p < 0.01$). See **Supplementary Table 2** for full list. Select genes with an absolute value average natural logFC >0.35 are colored and labeled. The y-axis represents the -log10 of Bonferroni corrected *P* values, and the x-axis represents average natural logFC between conditions.
Extended Data Fig. 2	Identification of myeloid cell types and subclustering analysis of	ED_Fig2.pdf	a. tSNE plot shows myeloid cells (n=15,288) colored and labeled by cell type. mDC = mature dendritic cell. Mono.Macro = monocytes and macrophages.

	proinflammatory microglia		b. Dot plot shows top marker genes for each cell type ranked by average natural logFC. Dot size represents the percentage of cells that express the gene, and dot greyscale represents the average expression level. See **Supplementary Table 4**. c. Bar chart shows the frequency of cells contributed by each mouse that localize to each cell type in **b**. d. Feature plots show myeloid cells colored by canonical cell type marker genes or features. Stressed cells were identified by increased expression of mitochondrial genome (percent.mito) genes, and decreased number of genes detected (nFeature_RNA). e. Bayesian information criterion (BIC) for microglia topic models from **Figure 2d** with the listed number of topics (K), each fit to an error tol = 10. f. Bar plot shows the relative enrichment of each topic in control and metastatic animals from **Figure 2d**. The relative enrichment was determined by subtracting the

			average topic assignment for the control mice from the average topic assignment across all cells in each mouse. Highlighted topics show four core topics where all three metastatic mice have a higher relative enrichment than all three control mice (i.e. min(Metastatic) > max(Control)).
Extended Data Fig. 3	Pro-inflammatory marker expression in microglia from BCBM models	ED_Fig3.pdf	a. Gating strategy for identification of microglia. Dot plots (top) show gating for single, live (zombie negative) $CD45^{lo}CD11b^{+}Ly6C^{-}$ microglia. Histogram plots (bottom) show subsequent gating for CD74, BST2, and MHC-II in microglia. b. Flow cytometry analysis of CD74, BST2 and MHC-II in microglia harvested 14 days post intracardiac injection of 4T1-GFP (100,000) cells into BALB/c animals. Bar graph shows the percent of microglia that express each marker in control (n=7) and metastatic (n=7) brains. *P* values were generated by an unpaired two-

			sided student's *t*-test, and error bars indicate mean +/- standard deviation. c. Quantification of microglia in tumor and distal regions of mice bearing EO771-GFP tumors. Representative images (left panels) show microglia localization relative to other cell types using a machine learning classifier (see Methods). Pie graphs (right panels) show the proportion of microglia and other cell types in each region. Frequencies are as follows: other non-microglia cells ($TMEM119^-CD74^-MHC\text{-}II^-ISG15^-$), distal =0.94, tumor =0.22; microglia ($TMEM119^+$), distal =0.05, tumor = 0.22; tumor cells ($ISG15^+TMEM119^-$), distal < 0.01 tumor = 0.14; other immune cells ($TMEM119^-CD74^+MHC\text{-}II^+$), distal < 0.01, tumor = 0.41. Scale bar= 100um.
Extended Data Fig. 4	Quantification of tumor size in FIRE-	ED_Fig4.pdf	a. IVIS images show EO771 luciferase luminescence signal change over time in FIRE-WT and FIRE-KO animals. Representative animals that displayed

	WT and FIRE-KO animals		continuous signal increase (tumor growth, solid line) vs. signal decrease (tumor rejection, dashed line) are shown. Pseudocoloring of luminescence shows quantification of radiance (p/sec/cm^2/sr). b. Line graphs show quantification of luminescence signal change over time in all FIRE-WT and FIRE-KO animals. Solid lines indicate animals that demonstrated tumor growth and dashed lines indicate those that showed tumor rejection. Growth was defined by signal increase over time, and rejection was defined as either baseline signal ($<10^6$) or >5-fold decrease in signal relative to maximum. c. Serial dilution analysis of EO771 cell engraftment in FIRE-WT and FIRE-KO animals. 10-200 x 10^4 EO771 cells were transplanted intracranially into each mouse strain. Ex vivo whole brain luminescence images show signal from tumor cells in each tissue at day 14.

			Fractions denote the number of grafts that produced macroscopic tumors in each condition. d. Dot plots quantify luminescent signal (total flux) from each tissue shown in **Extended Data Figure 4c** at day 14. *P* values were generated by an unpaired two-sided student's *t*-test.
Extended Data Fig. 5	Analysis of NK, T, and monocyte responses to BCBM in FIRE-WT and FIRE-KO animals	ED_Fig5.pdf	a. Quantification of tumor burden in FIRE-WT and FIRE-KO animals (n=8/group). Mice were injected with EO771 GFP-Luc cells as described in **Figure 5a** and tumors were harvested and analyzed by IVIS on day 7. Images (left panels) show pseudocoloring of radiance (p/sec/cm^2/sr), and bar graph shows quantification of total flux (p/s). *P* value shown is the result of a student's unpaired two sided *t*-test. Error bars represent mean +/- standard deviation. b. Box plots show frequency of T cell subsets from **Figure 5c** (n=7 FIRE-WT, n=8 FIRE-KO)**.** Frequencies shown are out of all T cells. Bounds of box and

			whiskers are indicative of the first through fourth interquartile range. *P* value shown is the result of a student's un-paired two sided *t*-test. c. Analysis of T cell activation in tumor bearing FIRE-WT and FIRE-KO brain tissues by flow cytometry. CD44 and CD62L expressions were measured in CD4 and CD8 T cells to delineate T effector (Teff), T central memory (TCM) and naive T cell subsets. Representative FACS plots (top panels) show gating for each subset after gating for single, live (Sytox negative) cells. Bar graphs (bottom panels) show quantification of T cell counts for each group. Error bars represent mean +/- standard deviation. Pairwise comparisons of counts between groups were not significant. d. Quantification of monocytes in tumor bearing FIRE-WT and FIRE-KO brain tissues by flow cytometry. $CD11b^{+}Ly6C^{+}$ monocytes were identified following

			gating for $CD3^{-}NK1.1^{-}$, single, live (Sytox negative) cells. Top panels show representative FACS plots, and bottom panels show quantification of cell counts. Error bars represent mean +/-standard deviation. Pairwise comparisons of counts between groups were not significant. e. Linear regression model of CD8+ T cell and Treg quantification from **Extended Data Figure 5b.** R-squared and *P* values determined by simple linear regression function.
Extended Data Fig. 6	Analysis of tumor burden and the immune response in T cell deficient mice	ED_Fig6.pdf	a. Gating scheme for analysis of T cells in brain tissue harvested from vehicle treated, FTY720 treated and RAG1-KO mice. FACS plots show gating of $TCRb^{+}$ T cells from single, live (sytox negative) $CD45^{hi}CD11b^{-}$ cells from **Extended Data Figure 6b**. b. Gating scheme for analysis of microglia and monocytes in brain tissue from vehicle, FTY720 and RAG1-KO animals. FACS plots (top panels) show

			gating for $CD45^{hi\text{-}int}$ and $CD11b^{+}$ cell populations, followed by gating for $CD45^{hi}Ly6C^{+}$ monocytes and $CD45^{int}Ly6C^{-}$ microglia (bottom panels). c. Bar graphs show the percentage of microglia and monocytes out of total live, single cells in brains harvested from vehicle (veh, n=6), FTY720 (FTY, n=6) and RAG1-KO (RAG1, n=4) animals. Pairwise comparisons of percentages between groups were not significant. Error bars represent standard deviation. d. Quantification of EO771 tumor burden at endpoint on day 12 by IVIS. Pseudocolor shows radiance ($p/sec/cm^2/sr$) in each whole brain.
Extended Data Fig. 7	Analysis of BCBM immune repertoire in T cell deficient and replete mice	ED_Fig7.pdf	a. Dot plot shows top marker genes for each cell type in total $CD45^{hi\text{-}int}$ sorted cells, ranked by the average log_2 fold-change and determined by the Wilcoxon rank sum test. Dot size represents the percentage of cells that express each gene, and dot greyscale represents the average expression level. Macro.DCs = macrophages

			and dendritic cells, mDCs = mature dendritic cells, pvMacro = perivascular macrophages. Bar graphs illustrate relative contribution of each cluster to total leukocytes, separated by mouse strain and timepoint. b. UMAPs show T cells (n=1949 cells) from C57BL/6 mice at day 4 and 10, colored by cluster (left) and timepoint (right). c. Bar graph illustrates the distribution of T cell clusters in each animal (n=6) separated by timepoint. d. Dot plot shows expression of top marker genes for each T cell cluster from **Extended Data Figure 7b**. CD4.eff = CD4+ effector T cell, CD8.eff = CD8+ effector T cell, Lt.stg.eff = late stage effector T cell, γδ = gamma delta T cell. e. Dot plot shows expression of top marker genes for each microglia cell cluster from **Figure 7b**.
Extended Data Fig. 8	Analysis of humanized mouse	ED_Fig8.pdf	a. Whole mount brightfield and fluorescence microscopy images show brains from MITRG mice transplanted

	model of BCBM and patient BCBM data		with GFP-labeled iHPSC cells and mCherry-labeled 231BR cells from **Figure 8a.** b. Identification of mouse and human cells by the frequency of reads that align to the mm10 mouse genome. Cutoffs used to identify mouse cells (>0.95 aligned, n=641 cells), human cells (<0.1 aligned, n=25,287 cells) and doublets (0.1-0.95 aligned, n=387 cells) are shown. c. Violin plots show cell distributions for key quality control metrics pre- and post- filtering and removal of poor quality cells. Cells were removed that displayed >20% of genes mapped to the mitochondrial genome (percent mito genes). d. tSNE plot shows human cells, colored by cluster and labeled by cell type. pvMacro=perivascular macrophages, Cycling = cycling myeloid cells. See **Supplementary Table 6** for full gene list.

			e. Dot plot shows top marker genes for each cell type determined by the Wilcoxon rank sum test and ranked by average natural logFC. Dot size represents the percentage of cells that express the gene, and dot greyscale represents the average expression level. See **Supplementary Table 6** for full gene list. pvMacro=perivascular macrophages. f. Bar chart shows the frequency of cells contributed by each mouse to the cell types shown in **e**. g. tSNE plots colored to show the expression of BST2, CD74 and CCL3. h. tSNE plot shows the distribution in human microglia of the three core topics identified in mouse microglia in response to BCBM. Gene scores for each topic from **Figure 2e** were generated using the AddModuleScore function in Seurat (See Methods). Each topic is indicated by color, where only cells with a topic score > 0.25 are colored. Contrast gray scale indicates topic

			weights. Scaling was performed by dividing all topic scores by the maximum topic score across the dataset. i. Bar plot shows the relative enrichment of each topic score in human microglia. j. Kaplan-Meier plots show overall survival probability stratified by MHC-II, CSF1, and BST2 expression in bulk RNA-seq data from human patient BCBM tumors (Varešlija et al, 2018).

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Item	Present?	Filename Whole original file name including extension. i.e.: Smith_SI.pdf. The extension must be .pdf	A brief, numerical description of file contents. i.e.: *Supplementary Figures 1-4, Supplementary Discussion, and Supplementary Tables 1-4.*
Supplementary Information	Yes	FACS gating strategies.pdf	FACS gating strategies
Reporting Summary	Yes	[F] NCB-L45274D	

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Supplementary Table	Supplementary tables 1.xlsx	Supplementary tables	Supplementary tables 1-6

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Parent Figure or Table	Filename Whole original file name including extension. i.e.: *Smith_SourceData_Fig1.xls,* or *Smith_Unmodified_Gels_Fig1.pdf*	Data description i.e.: Unprocessed western Blots and/or gels, Statistical Source Data, etc.
Source Data Fig. 1	Source_Fig1.xlsx	Quantification of immunofluorescence images

Source Data Fig. 3	Source_Fig3.xlsx	Quantification of flow cytometry data and cytokine array
Source Data Fig. 4	Source_Fig4.xlsx	Quantification of tumor burden in FIRE mice
Source Data Fig. 5	Source_Fig5.xlsx	Quantification of flow cytometry data
Source Data Fig. 6	Source_Fig6.xlsx	Quantification of flow cytometry and IVIS data
Source Data Extended Data Fig./Table 3	Source_ED_Fig3.xlsx	Quantification of flow cytometry data
Source Data Extended Data Fig./Table 4	Source_ED_Fig4.xlsx	Quantification of IVIS data
Source Data Extended Data Fig./Table 5	Source_ED_Fig5.xlsx	Quantification of flow cytometry and IVIS data
Source Data Extended Data Fig./Table 6	Source_ED_Fig6.xlsx	Quantification of flow cytometry data

Microglia promote anti-tumor immunity and suppress breast cancer brain metastasis

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Keywords

Breast cancer, brain metastasis, single cell, RNA sequencing, microglia, macrophages, tumor associated macrophage, TAM, FIRE, *Csf1r*$^{\Delta FIRE/\Delta FIRE}$

Abstract

Breast cancer brain metastasis (BCBM) is a lethal disease with no effective treatments. Prior work has shown that brain cancers and metastases are densely infiltrated with anti-inflammatory, protumorigenic tumor associated macrophages (TAMs), but the role of brain resident microglia remains controversial because they are challenging to discriminate from other TAMs. Using single-cell RNA-sequencing (scRNA-seq), genetic, and humanized mouse models, we specifically identify microglia and find that they play a distinct pro-inflammatory and tumor suppressive role in BCBM. Animals lacking microglia show increased metastasis, decreased survival, and reduced NK and T cell responses, showing that microglia are critical to promote antitumor immunity to suppress BCBM. We find that the pro-inflammatory response is conserved in human microglia, and markers of their response are associated with better prognosis in BCBM patients. These findings establish an important role for microglia in anti-tumor immunity and highlight them as a potential immunotherapy target for brain metastasis.

Introduction

Breast cancer brain metastasis (BCBM) is rapidly emerging as a critical problem in breast cancer. 15-30% of metastatic breast cancer patients develop brain metastasis, and studies project a >30% increase in patients as treatments for peripheral disease improve and patients live longer[1,2]. This is alarming since there are no effective treatments and median survival is only a few months[3–6]. There is growing interest in immunotherapeutic strategies to treat central nervous system (CNS) cancers, given that immune cells enter the brain while most conventional therapies are excluded by the blood brain barrier[7,8]. Greater understanding of the immune response to BCBM will be needed to develop immunotherapy strategies effective in the unique immune microenvironment of the CNS.

The brain immune microenvironment is principally composed of specialized tissue resident macrophages called microglia that tile the brain and play diverse functions in CNS homeostasis and disease[9–11]. Microglia represent an attractive immunotherapeutic target because they are the first line of defense to disease in the CNS. BCBMs are heavily infiltrated with tumor associated macrophages (TAMs), which may be comprised of microglia, macrophages and bone marrow derived myeloid cells[12–16]. Functional studies suggest a tumor promoting role for TAMs. Depletion of TAMs with CSF1R inhibitors results in tumor reduction and decreased metastasis in glioblastoma and melanoma models[17–21]. TAM depletion using a CX3CR1-targeted genetic ablation model similarly results in decreased BCBM[22]. However, it is unclear whether microglia or other TAMs produce these tumor promoting effects. CSF1R inhibitors deplete microglia but also attenuate other myeloid cells, and microglia ultimately repopulate the brain when treatment ceases. Likewise, CX3CR1 is expressed by diverse myeloid cell populations and upregulated by myeloid cells upon entry into the brain[23]. Therefore, the specific impact of microglia on tumor initiation and their potential as an immunotherapy target remain unclear.

We combined single cell RNA-sequencing (scRNA-seq) with genetic and humanized mouse models to find that microglia suppress BCBM by promoting anti-tumor NK and T cell

responses. ScRNA-seq of >90,000 cells from three different BCBM models revealed that microglia mount a robust pro-inflammatory response to BCBM. Using a genetic knockout model, we find that animals lacking microglia show increased metastasis, decreased survival, and impaired NK and T cell responses to BCBM[24]. We show that ablation of T cells reduces microglia activation and attenuates their tumor suppressive effect, indicating that reciprocal microglia-T cell activation is critical to suppress BCBM. Utilizing a humanized mouse model, we find that the pro-inflammatory response is conserved in human microglia, and analysis of patient BCBM data indicates that increased pro-inflammatory marker expression is associated with better prognosis. These findings contrast with the pro-tumorigenic function reported for TAMs and highlight the potential of harnessing the anti-tumor function of microglia to treat brain metastasis.

Results

Single-cell analysis of TAMs in BCBM

We used scRNA-seq to interrogate the microglia response to BCBM using the MDA-MB-231-BR (231BR) model. In this model, GFP-labeled 231BR cells are delivered into the arterial circulation via intracardiac injection and form parenchymal brain metastases by day 28 (**Fig 1a,b, Extended Data Figure 1a**)[26,27,28,29]. Like in human BCBM, metastases are heavily infiltrated with IBA1+ (ionized calcium-binding adaptor molecule 1) TAMs **(Fig 1b,c, Extended Data Figure 1b)**.[25] For scRNA-seq, cells were dissociated from control and metastatic brains and myeloid cells were isolated by flow cytometry (**Fig 1d, Extended Data Figure 1c**). Cancer cells and astrocytes were sorted for control (**Extended Data Figure 1c, Fig 1d**). Analysis of the 42,891 cells that passed quality control filtering (**Extended Data Figure 1d,e**) revealed seven distinct cell types identified by canonical markers **(Fig 1e,f, Supplementary Table 1)**. This included the targeted cell types, astrocytes (*Aldoc, Atp1a2*), microglia (*Tmem119, P2ry12*) and non-microglia myeloid cells (*Lyz2, Plac8*) (**Fig 1e,f**, **Supplementary Table 1**). We also recovered small numbers of ependymal cells (*Ccdc153, Rarres2*), oligodendrocytes (*Mbp, Ptgds*), vascular cells (*Cldn5, Vtn*),

and lymphocytes (*Cd3g, Gzma*) (**Fig 1e,f, Supplementary Table 1**). Lymphocytes and the non-microglia myeloid populations were preferentially from the metastatic condition, suggesting these cells are recruited from the periphery (**Extended Data Figure 1f**). We found limited differences in clustering of astrocytes from control and metastatic brains **(Extended Data Figure 1g,h, Supplementary Table 2)**.

Microglia display robust pro-inflammatory responses to BCBM

In contrast to astrocytes, analysis of the myeloid cells revealed strong separation of control and metastatic conditions **(Fig 2a)**. Microglia were distinguished from other myeloid populations by scoring each cell for the core microglia signature developed by Bowman et al (2016) (**Fig 2b, Supplementary Table 3**)[30]. This identified two large microglia populations *(Tmem119, P2ry12, Sparc, Gpr34)*, where one contained microglia from both control and metastatic and the other was almost entirely from metastatic **(Fig 2a,b, Extended Data Figure 2a-d)**. We also found two small populations of microglia that display an increased stress response (**Extended Data Figure 2d)**, which is common post tissue manipulation[31]. Neutrophils (*Camp*, *S100a9*), monocytes/macrophages (*Ly6c2, Lyz2*), mature dendritic cells (*Ccr7, Flt3*), and B cells (*Igkc, Cd79a*) were also identified (**Fig 2b, Extended Data Figure 2a-d, Supplementary Table 4**).

Further analysis of the microglia revealed robust changes in BCBM. We identified 3,715 genes differentially expressed between microglia from control and metastatic brains (**Supplementary Table 4**). Gene Ontology (GO) analysis showed that top upregulated pathways were associated with pro-inflammatory responses, such as ‘cytokine production,’ ‘antigen processing and presentation’ and ‘response to IFN-beta’ (**Fig 2c**)[32]. Further analysis revealed that these programs are not uniformly upregulated by all microglia. We used a probabilistic clustering method called latent dirichlet allocation (LDA), also known as topic modeling, to assess microglia heterogeneity (**Fig 2d**). Unlike standard cell clustering methods, topic modeling assigns each cell to multiple gene modules or topics, which allows for better appreciation of how distinct but

overlapping gene modules are expressed in a population of cells[33].This analysis identified four core topics (**Extended Data Figure 2e-f, Supplementary Table 5**). Topic 12 was the most broadly upregulated and represented an interferon (IFN) response program (*Bst2, Ifitm3, Ifit3b, Isg15*), which has been previously reported by microglia in other disease contexts[34],[35],[36] (**Fig 2e-g**). This likely represents the initial sensing of microglia to metastatic infiltration and tissue damage[37]. Topic 15 showed a more restricted expression pattern and was enriched for genes associated with antigen presentation (AP) (*Cd74, H2-Aa, H2-D1*) (**Fig 2e-g**), which has also been observed in glioma and Alzheimer's Disease (AD)[34,36]. AP genes enable antigen presentation to T cells, raising the question of whether microglia present antigen to T cells in the CNS. Topic 14 was expressed by a small subset of microglia and was associated with a secretory phenotype (**Fig 2e-g**). This topic was enriched for genes associated with exosomes (*Cd63*), lipid metabolism (*Apoe, Lpl*), and cytokines (*Spp1, Csf1, Il1b, Tnf)* (**Fig 2e,g**). This topic strongly overlaps with the signature of disease-associated microglia or "DAMs", a population of phagocytic microglia identified in neurodegeneration[38]. The IFN response and AP topics both included genes encoding numerous chemokines for immune cell trafficking (**Fig 2e,g, Supplementary Table 5**). A final topic (topic 3) was enriched for ribosomal genes (**Extended Data Figure 2f, Supplementary Table 5**), which could indicate cells with increased transcriptional capacity or stress response. These data show that microglia upregulate multiple pro-inflammatory programs, suggesting they play diverse roles in the immune response to BCBM.

The microglia response is conserved in diverse BCBM models

We validated the microglia pro-inflammatory response at the protein level by flow cytometry, in situ immunofluorescence (IF) and cytokine array. We evaluated three key markers by flow cytometry: bone marrow stromal antigen 2 (BST2), major histocompatibility complex II (MHC-II) and CD74. We found increased expression of each marker was conserved in five different BCBM models **(Fig 3a, Extended Data Figure 3a,b)**.

We used a multiplex IF system (co-detection by indexing, CODEX) for in situ validation. We co-stained for MHC-II, CD74, and IFN-stimulated gene 15 (ISG15), as well as TMEM119 and GFP to identify microglia and tumor cells, respectively. We found that pro-inflammatory microglia localize proximal to tumor cells, while distal microglia are negative **(Fig 3b,c, Extended Data Figure 3c).** The highest frequency of microglia co-expressed all three markers (MHC-II^{+}CD74^{+}ISG15^{+}, 29%) **(Fig 3c).** We also observed subpopulations of microglia that express only the AP markers (MHC-II+CD74+ISG15^{-}, 11%) or the IFN response marker (MHC-II-CD74^{-}ISG15^{+}, 11%) **(Fig 3c)**. These data are consistent with our topic modeling, showing substantial marker overlap but notable exclusivity of the AP and IFN response programs into different subsets of microglia.

We investigated the pro-inflammatory function of microglia using a cytokine array. Consistent with our scRNA-seq, we found that microglia from tumor-bearing brains upregulate several pro-inflammatory cytokines, including macrophage colony-stimulating factor (CSF1), chemokine ligand 5 (CCL5), chemokine ligand 9 (CXCL9) and chemokine ligand 10 (CXCL10) **(Fig 3d)**[47,48]. Taken together, these data validate our scRNA-seq results at the protein level and demonstrate that microglia display a pro-inflammatory response to BCBM.

Animals lacking microglia show increased tumor progression

Prior work established a pro-tumorigenic role for TAMs in brain cancers and metastases[19–22]. These studies primarily utilized CSF1R inhibitors and CX3CR1-targeted genetic ablation strategies that target microglia and other types of TAMs[20,22,49,50]. A genetic model was recently developed that specifically lacks microglia due to deletion of a key super-enhancer in the *Csf1r* locus called the fms-intronic regulatory element (FIRE) (**Fig 4a**)[24]. The *Csf1r*$^{\Delta FIRE/\Delta FIRE}$ (FIRE-KO) model lacks microglia while retaining most brain resident macrophages and bone marrow derived myeloid cells, which we confirmed by flow cytometry **(Fig 4b)**[24,51]. We investigated the role of microglia in BCBM by comparing tumor progression in FIRE-WT and FIRE-KO animals. Mice

were injected with GFP and luciferase-labeled EO771 cells and monitored by *in vivo* bioluminescence (IVIS) (**Fig 4c**). Surprisingly, many FIRE-KO mice quickly developed overt clinical symptoms of advanced disease (**Fig 4d,e**). Five of 14 FIRE-KO mice died before endpoint (36% mortality), while all 19 FIRE-WT survived (0% mortality) (**Fig 4d**). Surviving FIRE-KO mice also displayed >20% decrease in body mass compared to FIRE-WT, indicating increased morbidity (**Fig 4e**). IVIS imaging revealed differences in the kinetics of tumor growth over time (**Extended Data Figure 4a,b**). We observed tumor rejection in eight of 19 FIRE-WT mice, while signal continued to increase in all 14 FIRE-KO animals (**Fig 4f, Extended Data Figure 4a,b**). We further compared tumor engraftment in FIRE-KO and FIRE-WT mice using a serial dilution approach. This showed increased engraftment efficiency and larger tumor growth in FIRE-KO compared to FIRE-WT mice (**Extended Data Figure 4c,d**). Together, these data show that animals lacking microglia demonstrate increased tumor growth and engraftment, and decreased capacity for tumor rejection.

Microglia promote NK and T cell responses to BCBM

Given the reduced tumor rejection we observed in FIRE-KO mice, we hypothesized that microglia promote tumor rejection through T cells. We tested this hypothesis by determining whether FIRE-KO mice show a reduced T cell response to BCBM. We injected EO771 cells into FIRE-WT and FIRE-KO animals and compared the number and frequency of NK, T, and myeloid cell populations by flow cytometry in the brain on day 7 when we begin to observe tumor rejection **(Fig 5a, Extended Data Figure 4b)**. Although ex vivo analysis showed no significant difference in tumor size at this timepoint **(Extended Data Figure 5a)**, FIRE-KO mice had reduced numbers and frequencies of all T cell subsets, including $CD4^+$, $CD8^+$ and T regulatory (Treg) cells **(Fig 5b,c, Extended Data Figure 5b**). NK and NKT cells were almost completely absent in FIRE-KO animals **(Fig 5b,c)**. Analysis of functional markers showed consistent reductions in the numbers of $CD8^+$ and $CD4^+$ effectors and central memory T cells in FIRE-KO **(Extended Data Figure 5c)**.

We also found a significant decrease in the number of degranulating CD107a$^+$ NK and CD8$^+$ cells in FIRE-KO **(Fig 5d)**. Analysis of CD11b$^+$Ly6c$^+$ monocytes showed no significant difference in their numbers between FIRE-WT and FIRE-KO **(Extended Data Figure 5d)**. Further analysis revealed that the CD8$^+$ T cell frequency negatively correlates with Tregs in FIRE-WT, but positively in FIRE-KO **(Extended Data Figure 5e)**. This means that in FIRE-WT, mice with more CD8$^+$ T cells have fewer Tregs, while in FIRE-KO, mice with more CD8$^+$ T cells also have more Tregs. Thus, in the absence of microglia, the CD8$^+$ T cells may be less effective at inducing tumor rejection because there are relatively more immunosuppressive Tregs. In sum, these data suggest that microglia promote an anti-tumor immune microenvironment through supporting NK, NKT, and T cell responses to BCBM.

Microglia and T cells coordinate the anti-tumor response

We further investigated whether the tumor suppressive effect of microglia is mediated through T cells by evaluating tumor growth in FIRE-WT animals lacking T cells. We used two approaches to target T cells, treatment with S1P inhibitors (FTY720) that block T cell trafficking to the CNS, and RAG1 KO mice that lack T cells **(Fig 6a)**. Animals were first injected with EO771 cells on day 0. FTY720 and vehicle were injected daily starting on day 0 until endpoint on day 12. Flow cytometry analysis confirmed T cell depletion in the brain using both approaches **(Fig 6b, Extended Data Figure 6a)**. The frequencies of microglia and monocytes were not significantly different among the groups **(Extended Data Figure 6b,c).** In both FTY720 and RAG1-KO groups, we found increased tumor engraftment and tumor burden relative to control animals **(Fig 6c,d, Extended Data Figure 6d).** This shows that microglia replete animals are less able to suppress BCBM in the absence of T cells, suggesting that microglia suppress BCBM at least in part through supporting the T cell response.

We also found interesting differences in microglia marker expression between wildtype and T cell deficient animals. Microglia from FTY720 treated mice showed 3.1- and 1.6-fold lower

percentages of the AP markers MHC-II+ (p=0.0400) and CD74+ (p=0.0271), respectively **(Fig 6e)**. The reduced expression of these proteins was even more pronounced in RAG1-KO mice **(Fig 6e)**, indicating that this is not simply an effect of FTY720 treatment. Furthermore, FTY720 treated and RAG1-KO mice had a 2-fold (p=0.0160) and 9.4-fold (p<0.0001) higher percentage of microglia positive for the IFN response protein BST2, respectively **(Fig 6e)**. This suggests that T cells may be required to fully license microglia to upregulate the AP program, and without T cells, microglia are limited to the IFN response program.

Altered microglia activation in animals lacking T cells

We used scRNA-seq to determine whether microglia upregulation of the AP program is dependent upon T cell infiltration. We transplanted EO771 cells into C57BL/6 and RAG1-KO mice and evaluated gene expression in sorted immune cells at two timepoints, day 4 and 10 **(Fig 7a)**. Clustering and marker gene analysis identified nine major immune cell types **(Fig 7b, Extended Data Figure 7a)**. Subset analysis revealed a >2-fold increase in the frequency of T cells from days 4 to 10 **(Extended Data Figure 7a)**, indicating increasing T cell infiltration with tumor progression. No T cells were detected from RAG1 KO mice **(Extended Data Figure 7a)**. We also found a robust expansion of T cell diversity from day 4 to 10 **(Extended Data Figure 7b-d)**. Most notable was the decrease in naïve T cells and increase in proliferating and CD8 effector T cells, showing that the relative frequency of activated T cells increases in the brain over time **(Extended Data Figure 7b-d)**.

We performed subset analysis of microglia to determine how their gene expression changes over time in parallel with T cell activation **(Fig 7b, Extended Data Figure 7e)**. Subclustering of microglia showed similar populations as we previously identified **(Fig 7b)**. We scored each microglia in the dataset for expression of top genes associated with each topic **(Fig 7c, Supplementary Table 3)**, IFN response, secretory, and AP. This showed that all three programs increase over time from day 4 to 10 in C57BL/6 animals. At day 4, we observed the

highest mean score for the secretory program and lowest for the AP program, suggesting more microglia express the secretory than AP program at the early timepoint **(Fig 7c)**. We found limited to no expression of the AP program in microglia from RAG1 KO mice that lack T cells **(Fig 7c-f)**. In contrast, we found similar expression of the secretory and IFN response programs in C57BL/6 and RAG1 KO mice, indicating that the AP but not secretory and IFN response programs are dependent on lymphocytes. Top markers of each program showed a similar pattern, where large numbers of microglia express CD63 (secretory) and BST2 (IFN response) at day 4 but limited microglia express CD74 (AP) **(Fig 7d-f)**. Pseudotemporal analysis (Monocle) suggested that microglia follow a progression from homeostatic, through the secretory and IFN response, ending in the AP and cycling clusters (**Fig 7g**). These data support a model where microglia initially upregulate the secretory and IFN response programs in response to cancer cell appearance in the brain, followed by upregulation of genes for antigen presentation after lymphocyte infiltration. This may serve to sustain T cell activation locally in the brain and explain why microglia loss results in a diminished T cell response. Of note, we also observe an expansion of immune suppressive cells (Tregs and monocytes) at the later timepoint **(Extended Data Figure 7a,c)**, which may counteract anti-tumor immunity and explain why tumors continue to grow in some animals.

The pro-inflammatory response is conserved in human microglia

We investigated the pro-inflammatory response in human microglia and its relevance in BCBM patients. We developed a humanized mouse model of BCBM based on prior work, where MITRG mice (human *CSF1*, *IL3* and *TPO* knock in to *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice) are reconstituted with human microglia and macrophages following transplantation of human induced pluripotency-derived hematopoietic progenitor cells (iHPSCs) into the postnatal brain[52–54]. In contrast to patient BCBM samples, we were able to use these animals to investigate the initial response of human microglia to tumor initiation. We injected MITRG mouse pups with GFP-labeled iHPSCs, allowed

engraftment for 10 weeks, and injected mCherry-labeled 231BR cells intracardially **(Fig 8a).** Control and metastatic mice were harvested three weeks later, and fluorescence microscopy confirmed the engraftment of GFP^+ human microglia and $mCherry^+$ 231BR metastases **(Extended Data Figure 8a)**. Human cells were subsequently isolated and captured for sequencing **(Fig 8a)**.

Clustering and marker gene analysis revealed a distinct population of 231BR cells *(VIM)* and several populations of myeloid cells (**Fig 8b, Extended Data Figure 8b-f, Supplementary Table 6**). These included human perivascular macrophages (*CD163*), microglia (*TMEM119, P2RY12)*, and a population of proliferating myeloid cells (*MKI67*) (**Fig8b**, **Extended Data Figure 8d-f, Supplementary Table 6**). We identified 4,904 genes differentially expressed between microglia from control and metastatic brains (**Supplementary Table 6**). Gene Ontology (GO) analysis revealed that similar pathways were upregulated in human microglia as observed in mouse (**Fig 8c**). We used gene scoring to investigate human microglia heterogeneity and expression of the core topics upregulated in mouse BCBM (**Fig 2d-f, Supplementary Table 3)**. This showed distinct but overlapping expression of the IFN response, AP, and secretory programs in subsets of human microglia like observed in mouse (**Extended Data Figure 8g-i**). Importantly, upregulation of the IFN response and AP topics in this model was not as robust as observed in mouse microglia (**Extended Data Figure 8i**). This is consistent with the more severe immune defects in MITRG mice and our findings that T cells are important for microglia activation.

We compared the prognostic relevance of microglia signatures in BCBM patients using a bulk RNA sequencing dataset of human BCBM tumors[55]. We found that patients with a high expression of canonical microglia markers had significantly better overall survival, suggesting increased microglia infiltration is associated with better outcomes **(Fig 8d)**. We further found that higher expression of key genes characteristic of the AP (MHC-II) and secretory programs (CSF1) are associated with increased overall survival, while higher expression of the IFN response gene BST2 is associated with decreased survival **(Extended Data Figure 8j).** These data suggest that

the microglia pro-inflammatory response can be clinically beneficial in patients and support the hypothesis that activation of microglia by T cells (i.e., upregulation of the AP program) is a key feature of anti-tumor microglia, and incomplete activation (i.e., IFN response program only) leads to worse outcomes. In sum, our study supports a model where microglia are critical to support the anti-tumor immune response in the CNS and suppress BCBM **(Fig 8e)**.

Discussion

We utilized scRNA-seq, genetic and humanized mouse models to investigate the role of microglia in BCBM. Our scRNA-seq analyses revealed that mouse microglia upregulate three core pro-inflammatory programs in response to BCBM, IFN response, AP, and secretory. It is important to acknowledge the effects that tissue dissociation may have on the transcriptome, so we further validated key pro-inflammatory markers at the protein level in situ on undigested tissues.[56,57] Using the FIRE-KO model, we found that animals lacking microglia demonstrate less capacity for tumor regression due to reduced anti-tumor T and NK cell responses. T cell depletion experiments revealed altered microglia activation and less tumor regression in the absence of T cells, suggesting that reciprocal microglia-T cell activation is critical for tumor suppression. Finally, we used a humanized mouse model to show that human microglia upregulate similar pro-inflammatory response programs in response to BCBM and found that markers of antigen presentation by microglia are associated with better prognosis in BCBM patients, raising the prospect of targeting microglia to treat BCBM.

The pro-inflammatory, tumor suppressive role of microglia that we observe contrasts with the anti-inflammatory, pro-tumorigenic role previously ascribed to microglia and other TAMs. There are several possible explanations for these different results. Microglia depletion in the FIRE-KO model is more complete and restricted to microglia than other approaches[20,22,24,58]. Furthermore, microglia cannot rebound and repopulate the brain or become reprogrammed as

has been observed in other depletion models[59,60]. The massive cell death produced in the Cx3cr1$^{CreERT/+}$:ROSA26i$^{DTR/+}$ depletion model has also been shown to induce cytokine storm and astrogliosis, which may have confounding effects on tumor growth and the immune response[61,62]. Another important distinction is that the FIRE-KO mice lack microglia from birth, while most prior studies targeted TAMs postnatally and after tumor initiation. It is therefore plausible that the timing of depletion impacts the outcome, as microglia and TAMs may become tumor-promoting as disease progresses.

It will be important in future work to investigate the mechanism by which microglia support anti-tumor T cell responses in the CNS. We found that pro-inflammatory microglia secrete several chemokines that could promote T cell trafficking to the CNS, such as CCL5, CXCL9 and CXCL10 (**Fig 3d**). Pro-inflammatory microglia also upregulate AP machinery **(Fig 2)**, which could enable them to present tumor antigens to CD4 or CD8 T cells and sustain T cell activation locally in the brain. It will also be important to understand why microglia ultimately fail to control disease progression in the CNS to discover ways to reactivate CNS immunity in BCBM patients.

Acknowledgements

Research reported in this publication was supported by the National Institute of Health/ National Cancer Institute award number R01CA237376-01A1, to DAL., the American Cancer Society award number IRG-98-279-10 to DAL, the Center for Complex Biological Systems, University of California, Irvine NIH/NCI U54-CA-217378 to DAL and MBJ, the NIH/NINDS award number R01NS120060 to SAV, the National Institutes of Health T32AI 0602573, the Stanley Behrens Fellows in Medicine award and the UCI Graduate Dean's Dissertation Fellowship to KTE, the National Institute of Health award number NIHT32EB009418 and the UCI Public Impact Fellowship to KB, the National Institutes of Health award T32NS121727-01 to AL, the National Institute of Neurological Disorders, Stroke/ National

Institutes of Health under award number T32NS082174 to MAC, the Feodor-Lynen fellowship from the Alexander-von-Humboldt Stiftung to JIR, the Canadian Institutes of Health Research Postdoctoral Fellowship and the NIH/NCI K99/R00 Transition to Independence Award (1K99CA267160-01) to DM, and the National Institutes of Health T32GM136624 and F31CA281331 to TL. The authors wish to acknowledge the support of the Chao Family Comprehensive Cancer Center Experimental Tissue Shared Resource, supported by the National Cancer Institute of the National Institutes of Health under award number P30CA062203. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. Experiments using the GFP-expressing iPSC line AICS-0036 were made possible through the Allen Cell Collection, available from Coriell Institute for Medical Research. The authors also sincerely thank Othy Shivashankar for sharing their advice and expertise, Maren Pein for reviewing the manuscript, Delia Tifrea for obtaining human specimens, Jennifer Atwood for assistance and training in flow cytometry and cell sorting, Ian Smith for his generous gift of MDA-231-BR2 cells, Maren Pein for her generous gift of GFP and luciferase labeled EO771 cells, and Ivan Chang and the UCI Research Cyberinfrastructure Center for maintenance and development of computational resources. This research is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author contributions

Conceptualization, KTE, KB, AL, MAC, JIR, KNG, SAV, MBJ, and DAL; Methodology KTE, KB, AL, MAC;

Investigation, KTE, KB, AL, MAC, JIR, TM, QHN, DM, TL, GH, DO, and AKO; Resources, DAL, MBJ, CP and RAE; Writing - Original Draft, KTE, KB, AL and DAL; Writing - Review & Editing, DAL, CP and MBJ; Project Administration, DAL; Funding Acquisition, DAL, and MBJ.

Competing interests statement

The authors declare no competing interests.

Figure legends

Figure 1: Single-cell analysis of TAMs in BCBM

a. Schematic showing disease progression in mouse 231BR-Foxn1$^{nu/nu}$ BCBM experimental metastasis model. 500,000 GFP-Luc labeled 231BR cells were injected into the left cardiac ventricle of Foxn1$^{nu/nu}$ mice and harvested 28 days later. Whole mount brightfield and fluorescence microscopy images show a representative brain with GFP+ metastatic foci (green).

b. IF staining shows IBA1^{+} cells (red) in control and metastatic brains at 7, 14 and 28 days post 231BR cell injection. Metastatic cells are GFP^{+} (green). 231BR cells arrest in blood vessels and cross into the brain 2-7 days post-injection, then grow along blood vessels forming micrometastases by day 14, parenchymal metastases by day 28. Scale bar = 50 μm.

c. Quantification of IBA1^{+} cells in control (n=4) and metastatic (n=4) brains 28 days post 231BR cell injection. IBA1^{+} cells were quantified in control (n=61 fields) and metastatic tumor regions (n = 41 fields). Bar graph shows 1.95-fold increase of IBA1+ cells in tumor compared to control tissue. *P* value was generated using a two-sided, unpaired *t*-test and error bars show mean +/- standard deviation**.**

d. Schematic showing experimental design for generation of scRNA-seq dataset. Foxn1$^{nu/nu}$ mice were injected with 500,000 GFP-Luc labeled 231BR cells and brains were harvested 28 days later. Three metastatic (Met1-3) and three control (Con1-3) brains were digested and myeloid cells (CD45^{+}, CD11b^{+}), astrocytes (CD45^{-}, ASCA2^{+}) and 231BR (CD45^{-}, GFP^{+}) cells were isolated by flow cytometry for droplet based scRNA-seq.

e. tSNE plot shows mouse cells that passed quality filtering (n=42,891), colored and labeled by cell type.

f. Dot plot shows top marker genes for each cell type ranked by the average natural logFC and determined by the Wilcoxon rank sum test. Dot size represents the percentage of cells that express each gene, and dot greyscale represents the average expression level. See **Supplementary Table 1** for full marker gene list.

Figure 2: Microglia display robust pro-inflammatory responses to BCBM

a. tSNE plot shows clustering of myeloid cells (n=24,348), colored by mouse.

b. tSNE plot shows each myeloid cell colored by MG score, the core microglia gene signature from Bowman et al (2016) that compared microglia to bone marrow derived cells using bulk RNA sequencing from lineage labeled mice. See **Supplementary Table 3** for full MG score gene list. Scores were calculated using the AddModuleScore function in Seurat. Top marker genes (gray) for each myeloid cell type were identified using the Wilcoxon rank sum test. See **Supplementary Table 4** for myeloid cell type markers. mDC = mature dendritic cell; Mono/Macro = monocytes and macrophages.

c. Bar plot shows selected top gene ontology (GO) terms associated with the BCBM response microglia signature. This signature was generated by differential gene expression analysis of microglia from metastatic vs control brains (n=632 upregulated genes, adj. $p<0.05$). Differentially expressed (DE) genes were determined using the Wilcoxon rank sum test. GO terms were identified using MouseMine and select upregulated terms with Holm-Bonferroni adjusted *P* values <0.05 were retained. See **Supplementary Table 4** for DE genes.

d. Schematic overview of topic model fitting method to assess microglia heterogeneity. The CountClust R package was used to fit a topic model using Latent Dirichlet Allocation (LDA). A matrix for ‘gene weights’ was generated that contains a list of the genes comprising each topic and the gene weight. See **Supplementary Table 5.** A second matrix for ‘topic weights’ lists the weight of each topic across the cells.

e. Heatmap shows three core topics upregulated in microglia in BCBM. Scaled gene weights for top genes comprising each topic are shown.

f. tSNE plots show distribution of three core topics in each microglia. Left panels show topic weight in each cell indicated by contrast gray scale. Right panel overlay shows top topic assignment for each cell, where only cells with a topic weight > 0.1 are colored.

g. tSNE plots show expression of selected genes from each topic in myeloid cells.

Figure 3: The microglia pro-inflammatory response is conserved in diverse BCBM models

a. Flow cytometry analysis of CD74, BST2 and MHC-II expression in microglia from four BCBM models. Microglia were identified by gating on $CD45^{lo}$, $CD11b^{+}$, $Ly6C^{-}$ cells. Bar graphs show the percent of microglia expressing each marker. *P* values were generated by an unpaired two-sided student's *t*-test, error bars indicate mean +/- standard deviation.

b. In situ analysis of microglia pro-inflammatory marker expression by multiplex IF (CODEX). Brain tissue slices from mice bearing EO771-GFP tumors were stained for DAPI (blue), and antibodies against GFP (green), TMEM119 (red), MHC-II (white), CD74 (yellow), and ISG15 (cyan). Left panel shows overview of all markers. Scale bar = 840 microns. Right panels show pairwise marker expression in higher magnification insets of tumor and distal regions. Short arrows indicate representative microglia expressing AP markers ($TMEM119^{+}MHC\text{-}II^{+}CD74^{+}$), and long arrows indicate representative microglia expressing IFN response marker ($TMEM119^{+}ISG15^{+}$). Results are representative of two independent experiments. Scale bar = 100 microns.

c. Quantification of pro-inflammatory markers in brain tissue slices. Microglia were identified based on TMEM119 expression and then scored for marker expression. Images (left panels) show phenotype in representative tumor and distal regions. Tumor cells ($ISG15^{+}TMEM119^{-}$) and other non-microglia cells ($TMEM119^{-}CD74^{-}MHC\text{-}II^{-}ISG15^{-}$) are shown in green and gray,

respectively. Pie graphs (right panels) show the proportion of microglia displaying marker combinations. Frequencies are as follows: $CD74^{+}MHCII^{+}ISG15^{+}$, distal < 0.01, tumor = 0.29; $CD74^{+}MHC\text{-}II^{+}ISG15^{-}$, distal< 0.01, tumor =0.11; $CD74^{-}MHC\text{-}II^{+}ISG15^{+}$, distal < 0.01, tumor = 0.07; $CD74^{+}MHC\text{-}II^{-}ISG15^{+}$, distal < 0.01, tumor = 0.07; $CD74^{+}MHC\text{-}II^{-}ISG15^{-}$ distal = 0.01, tumor = 0.08; $CD74^{-}MHC\text{-}II^{+}ISG15^{-}$, distal <0.01, tumor = 0.02; $CD74^{-}MHC\text{-}II^{-}ISG15^{+}$, distal = 0.01, tumor =0.11; $CD74^{-}MHC\text{-}II^{-}ISG15$, distal = 0.97, tumor = 0.24. n=4 mice per condition. Scale bar = 100um.

d. Analysis of cytokine expression by microglia in BCBM. Microglia were isolated from control (n=4) and metastatic (EO771-C57BL/6, n=8) brains by flow cytometry, and cell lysates were analyzed by cytokine array (Eve technologies). *P* values shown are the result of a two-sided unpaired Mann-Whitney *t*-test.

Figure 4: Animals lacking microglia demonstrate reduced capacity for tumor rejection

a. Schematic depiction of *Csf1r*$^{\Delta FIRE/\Delta FIRE}$ mouse model. Deletion of FIRE super-enhancer in FIRE-KO mice leads to loss of CSF1R protein expression in specific tissues. In the CNS, microglia do not develop, while monocyte and macrophage numbers are unaffected.

b. Representative flow cytometry plots show the percentage of $CD45^{lo}CD11b^{+}$ microglia and $CD45^{hi}$ immune cells gated from live (sytox negative), single cells in FIRE-WT (n=2) and FIRE-KO (n=2) mouse brains.

c. Schematic of experimental design to compare disease progression in FIRE-WT and FIRE-KO mice. FIRE-WT (n=19) and FIRE-KO (n=14) mice were injected intracranially with 100,000 GFP and luciferase (GFP-Luc) labeled EO771 cells. Control FIRE-WT mice (n=8) were also injected with PBS. Animals were imaged for luminescence (IVIS) every three days before dissection at endpoint on day 14.

d. Kaplan-Meier plot shows survival in FIRE-WT (19/19, 100%) and FIRE-KO (9/14, 64%) mice. *P* value determined by log-rank (Mantel-Cox) test.

e. Bar graph shows percentage body weight change for surviving PBS injected (n= 8), FIRE-WT (n=19), and FIRE-KO (n=9) animal from **d** at day 14 relative to day 0. *P* values determined by unpaired two-sided student's *t*-test and error bars represent mean +/- standard deviation.

f. Bar graph summarizes the frequency of animals that displayed tumor growth and tumor rejection in FIRE-WT and FIRE-KO mice. Tumor rejection was defined by a lack of engraftment or engraftment followed by tumor rejection. *P* value was determined by two-sided Fisher's exact test.

Figure 5: Microglia promote NK and T cell responses to BCBM

a. Schematic of experimental design to compare NK and T cell responses in FIRE-WT and FIRE-KO EO771 tumor bearing mice. FIRE-WT (n=8) and FIRE-KO (n=8) mice were injected intracranially with 100,000 EO771 GFP-Luc cells. Animals were imaged for luminescence (IVIS) on day 1, 4 and 6 before dissection on day 7.

b. Analysis of NK and T cell subsets in FIRE-WT (n=7) and FIRE-KO (n=8) mice by flow cytometry. Representative FACS plots show gating for each NK and T cell subset after gating for single, live (Sytox negative) cells.

c. Bar graphs show cell counts for NK and T cell subsets. Counts shown are out of 100,000 single, live cells analyzed. *P* values are the result of a student's un-paired two sided *t*-test. Error bars represent mean +/- standard deviation.

d. Analysis of CD107a expression in NK and CD8+ T cells by flow cytometry. FACS plots (left panels) show expression of CD107a from spleen and brains of representative animals from each cohort. Bar graph shows cell counts out of 100,000 single, live cells. *P* values are the result of a student's un-paired two sided *t*-test. Error bars represent mean +/- standard deviation.

Figure 6: Microglia and T cells coordinate the anti-tumor response

a. Schematic of experimental design to determine effects of T cell deficiency on BCBM. Tumor burden was compared in three cohorts of animals, FIRE-WT vehicle treated (Veh., n=13), FIRE-WT FTY720 treated (FTY, n=6), and RAG1-KO (RAG1, n=12). Vehicle (PBS + 0.1% DMSO) or FTY720 (5mg/kg) were administered via intraperitoneal injection to FIRE-WT animals on day 0 and repeated daily. 70,000 EO771 GFP-Luc cells were delivered to each animal in all three cohorts by intracranial injection on day 0 following drug delivery. Brain tissues were harvested at endpoint on day 12 and analyzed for tumor burden by IVIS and immune response by flow cytometry.

b. Bar graph shows the percentage of $TCRb^{+}$ T cells in brain tissues harvested from each cohort (n=6 Veh., n=6 FTY, n=4 RAG1) of animals at endpoint, gated out of single, live (sytox negative) $CD45^{hi}$ cells as shown in **Extended Data Figure 6a,b**. *P* values shown are the result of an unpaired two sided student's *t*-test. Error bars represent mean +/- standard deviation.

c. Quantification of EO771 tumor engraftment at endpoint on day 12 by IVIS. Bar graph shows frequency of animals in vehicle (Veh.), FTY720 (FTY), and RAG1-KO (RAG1) groups that grew tumors. $P = 0.51$ Veh. vs FTY. and $P = 0.21$ Veh vs RAG1 by two-sided Fisher's exact test.

d. Quantification of EO771 tumor burden at endpoint on day 12 by IVIS. Box and whisker plots show total flux per brain of vehicle (Veh.) and FTY720 (FTY) injected FIRE-WT and RAG1-KO (RAG1) cohorts. Bounds of box and whiskers are indicative of the first through fourth interquartile range. *P* values shown are the result of an unpaired two sided student's *t*-test.

e. Analysis of pro-inflammatory marker expression in microglia from T cell deficient mice (n=6 Veh., n=6 FTY, n=4 RAG1). FACS plots (left panels) show expression of MHC-II, CD74 and BST2 in representative animals, following gating on single, live (sytox negative) $CD45^{int}CD11b^{+}Ly6c^{neg}$ microglia as shown in **Extended Data Figure 6b**. Bar graphs (right panels) show the percentage of marker positive microglia in each cohort. *P* values are the

result of an unpaired two sided student's *t*-test. Error bars represent mean +/- standard deviation.

Figure 7: Altered microglia activation in animals lacking T cells

a. Schematic of experimental design to evaluate changes in microglia and T cell activation over time. 100,000 EO771 GFP-Luc cells were administered through intracranial injection to C57BL/6 (n=6) and RAG1-KO (n=6) mice at day 0. Brain tissues were harvested 4 (n=3/group) and 10 days (n=3/group) post injection and sorted for live, $CD45^{hi\text{-}int}$ cells by flow cytometry for scRNA-seq analysis.

b. UMAPs show all immune cells (n=31,053 cells) (left), microglia colored by subcluster (middle), and microglia colored by condition (right). UMAPs for microglia were downsampled to display an equal number of microglia from each condition (n=1000 cells per condition).

c. Bar graph shows the mean topic score for each program (antigen presentation (AP), secretory, and IFN response) in all microglia from each condition.

d. Violin plots quantify the expression of key markers of the secretory, IFN response, and antigen presentation (AP) programs in microglia from each condition.

e. Feature plots illustrating the distribution of key markers of the secretory, IFN response and antigen presentation (AP) programs in microglia.

f. Heat map of $\log_2$ fold change of key markers of the secretory (Secr, top), IFN response (IFNR, middle), and antigen presentation (AP, bottom) programs separated by timepoint and mouse strain.

g. UMAP plot of pseudotemporal cell ordering results performed using Monocle 3 showing microglia cell state ordering beginning with the homeostatic state (pseudotime = 0). Violin plot shows the contribution of each microglia cluster at specific pseudotime values. Microglia cell states are ordered by the median pseudotime value displayed as a black bar.

Figure 8: The pro-inflammatory response is conserved in human microglia and associated with better prognosis in BCBM patients

a. Schematic shows experimental design for scRNA-seq of human microglia from humanized MITRG mice transplanted with 231BR cells. MITRG mouse pups were injected with GFP-labeled iHPSCs, aged to 10 weeks and injected intracardiac with mCherry-labeled 231BR cells. Brains from control (n=3) and metastatic (n=3) mice were digested to single cell suspensions three weeks later. Dissociated cells from each sample were indexed using MULTI-seq. Mouse cells were removed using anti-mouse MHC-I magnetic beads, and recovered cells were pooled into metastatic or control samples for scRNA-seq.

b. tSNE plot shows human cells (n=21,353) colored by mouse and labeled by cell type. Top marker genes (gray) for each cell type were identified using the Wilcoxon rank sum test. See **Supplementary Table 6** for full marker gene list. pvMacro=perivascular macrophages.

c. Bar plot shows selected top GO terms associated with the human BCBM microglia response signature. DE genes (n=4,904, adjusted p<0.05) were determined using the Wilcoxon rank sum test. GO terms were determined using Enrichr and select upregulated terms with *P* values <0.05 were retained. See **Supplementary Table 6** for full gene list.

d. Kaplan-Meier plot shows overall survival probability in human BCBM patients stratified by expression of canonical microglia genes. Bulk RNA-seq data from patient BCBM tumors (n=20, Varešlija et al, 2018)[55] was scored for microglia gene signature and stratified into high and low groups. Scores were determined using the sum of scaled and centered values from log(CPM + 1) transformed data.

e. Model for role of microglia in promoting anti-tumor immunity. In microglia replete conditions (+ Microglia), microglia respond to BCBM by upregulating proinflammatory programs (IFN response, antigen presentation (AP), and secretory) that promote anti-tumor CD4, CD8, and NK cell responses and tumor regression in the CNS. In microglia depleted conditions (- Microglia), NK and T cell responses are deficient and the proportion of Tregs is increased,

resulting in tumor progression. In animals lacking T cells (-T cells), microglia fail to upregulate AP genes and tumor regression is not observed, suggesting that T cells are required for complete microglia activation and that reciprocal microglia-T cell activation is critical for tumor suppression.

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Current concepts and future directions. *Mol. Brain* **10**, 1–8 (2017).

Methods

Research within this publication complies with relevant ethical regulations**.** Animal studies were performed in accordance with an IACUC approved protocol #AUP-19-051 at the University of California Irvine. Human FFPE samples were exempted by IRB as human subjects research due to patient deidentification.

Normal human brain and human BCBM samples

FFPE sections from four deidentified normal female human brain and resected breast cancer brain metastasis were acquired from University of California Irvine department of Pathology and Laboratory Medicine, experimental tissue shared resource facility and the University of California Davis Pathology Biorepository.

Cell lines

MDA-MB-231-Br2[26] cells stably transduced with membrane targeted AcGFP (rLV.EF1.AcGFP1Mem-9, ClonTech/Takara Bio, USA, Cat#0019VCT), mCherry (rLV.EF1.mCherry-9, ClonTech/Takara, Cat#0037VCT), and luciferase lentivirus were a gift from Ian Smith (Parker, 2017, Bos, 2009). 4T1 cells were purchased from ATCC (ATCC Cat# CRL-2539, RRID:CVCL_0125), stably infected with GFP lentivirus (Santa Cruz Biotechnology, copGFP Control Lentiviral Particles, Cat#sc-108084) at a MOI of10, and sorted for GFP expression .EO771 cells were purchased from CH3 Biosystems (Cat. No. 94A001, RRID:CVCL_GR23) and stably infected with pCDH-EF1a-eFFly-eGFP) lentivirus particles. pCDH-EF1a-eFFly-eGFP was a gift

from Irmela Jeremias (Addgene plasmid #104834; http://n2t.net/addgene:104834; RRID:Addgene_104834). To produce lentiviral particles, HEK293T cells were transfected with pCDH-EF1a-eFFly-eGFP together with pMD2G and psPAX2 packaging plasmids using Lipofectamine 2000 (Invitrogen, Cat# 11668027). Supernatants containing lentiviral particles were used to infect EO771 cells overnight in the presence of 8 µg/ml polybrene (Sigma-Aldrich, Cat# TR-1003-G). Transduced EO771 cells were sorted by GFP expression on a BD FACSAria Fusion cell sorter. MDA213-BRm and4T1 cell lines were cultured in DMEM, 5% FBS, 10U/ml penicillin, 0.1mg/mL streptomycin (GE Healthcare Cat#SV30010), at 37 °C, 5% CO_2, 95% relative humidity. EO771 cells were cultured in RPMI 1640, 5% FBS, 10U/ml penicillin, 0.1mg/mL streptomycin, 10mmol/L HEPES at 37 °C, 5% CO_2, 95% relative humidity. All cell lines were authenticated by STR analysis by ATCC prior to injection.

Mouse strains

Female Foxn1$^{nu/nu}$ mice (IMSR Cat# JAX:007850, RRID: IMSR_JAX:007850), FVB (IMSR Cat# JAX:001800, RRID:IMSR JAX:001800), C57BL/6J (IMSR Cat# JAX:000664, RRID: IMSR_JAX:000664), B6(cg)-Tyrc-2J/J (IMSR Cat# JAX:000058, RRID:IMSR JAX: 000058, albino B6), BALB/cJ (IMSR Cat# JAX:000651, RRID:IMSR_JAX:000651) and B6.129S7-Rag1$^{tm1Mom/J}$ (RAG1-KO) were purchased from The Jackson Laboratories. Female MITRG mice (IMSR Cat# JAX:017711, RRID: CVCL_JM19) which are C:129S2- Rag2$^{tm1.1Flv}$ Csf1$^{tm1(CSF1)Flv}$ CSF2/IL3$_{tm1.1(CSF2,IL3)Flv}$ Thpo$_{tm1.1(TPO)Flv}$ Il2rg$_{tm1.1Flv}$/J were bred, housed and maintained by the laboratory of Mathew Blurton-Jones (IACUC protocol #AUP-17-162). *Csf1r*$^{\Delta FIRE/\Delta FIRE}$ (FIRE-KO) and *Csf1r*$^{FIRE/FIRE}$ (FIRE-WT) mice were a gift from Claire Pridans and Mathew Blurton-Jones laboratories and were housed and maintained by the Lawson laboratory. All animals were aged between 5-15 weeks old. Only female animals were included in these studies because breast cancer predominantly afflicts women.

Immunofluorescence analysis of human BCBM samples

4-µm sections were heated at 65 °C and deparaffinized in Histo-Clear (National Diagnostics, #HS-200, Atlanta, Georgia, USA). Tissues were rehydrated with graded solutions of ethanol (100%-50%). Antigen retrieval was performed using a microwave pressure cooker with 10 mM citric acid buffer (0.05% Tween-20, ThermoFisher Scientific Cat#BP337500, pH 6.0). Tissues were blocked in blocking solution (0.1% Tween-20 and 10% Goat Serum in PBS), incubated with primary antibodies diluted in blocking solution at 4 °C overnight, washed in PBS, incubated with secondary antibodies diluted in blocking solution for one hour at room temperature. Slides were mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, #H-1200, Burlingame, California, USA) and micrographs were taken with the BZ-X700 Keyence fluorescence microscope.

Generation of BCBM in mice

Intracardiac injections were performed as described by Campbell et al, 2012[63] into the left cardiac ventricle of anesthetized mice (300mg/kg Avertin). For 231BR brain metastasis 500,000 cells in 100µL of DPBS were injected into nine-week-old Foxn1$^{nu/nu}$ or 10-week-old MITRG mice. For 4T1 brain metastasis, 100,000 cells were injected into nine-week-old BALB/cJ mice in 100µL of DPBS. For the intracranial injection of FVB, C57BL/6, FIRE-WT, FIRE-KO, RAG1-KO or albino B6, 100,000 VO-PyMT, EO771, or Py8119 cells were injected in 10 µL PBS to a depth of 3mm into the right coronal suture of five-week-old mice[18,20]. Tumors were not allowed to exceed 1.7mm along any diameter or 10% of the animal's body weight in accordance with IACUC protocol. Control mice were injected with 10 µL PBS. Injections were replicated in 2-3 cohorts of 4-6 mice and in different mouse strains to ensure reproducibility of results.

Dissection and visualization of mouse BCBM by whole mount fluorescence microscopy

Mice were euthanized and perfused with 50mL of sterile ice cold 1X PBS, 1mg/mL EDTA. The brain was dissected from the cranium and meningesand whole brain metastasis was visualized on a dissection microscope (Leica Biosystems, DMC 2900) and imaged for GFP fluorescence and brightfield.

Mouse brain fixation and sectioning

Dissected brains were drop fixed into 4% PFA, 1X PBS, pH 7.4 overnight at 4°Cand transferred into 30% Sucrose 1X PBS for 24 hours prior to cryosectioning on sliding microtome (Leica Biosystems, SM2010R). Serial 40µm slices were collected into 1X PBS, 0.05% sodium azide and stored at 4°C for floating section immunostaining.

Immunofluorescence staining of floating sections

Brain slices were blocked (1X PBS, 5% serum, 0.3% tritonX-100) and placed on an orbital shaker for one hour. Blocking solution was replaced with 500µL of primary antibody in blocking solution and incubated overnight at 4°C. Brain slices were washed and incubated with secondary antibody for one hour at room temperature. Brain slices were slide-mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, # H-1200, Burlingame, California, USA). Micrographs were taken with the BZ-X700 Keyence fluorescence microscope and acquisition software. Primary antibodies: Rabbit polyclonal anti-IBA1 diluted 1:500 (RRID: AB_A39504 Wako Cat#019-19741); Secondary antibodies diluted 1:400: Goat anti-rabbit IgG conjugated with Alexa Fluor 568 and 488 (ThermoFisher Scientific, RRID: AB_2535730 Cat#A21069 and RRID: AB_2576217
Cat#A11034); Goat anti-rat IgG conjugated with Alexa Fluor 568 and 647 (RRID: AB_2534074

Cat#A11006and RRID: AB_141778 Cat#A21247); Goat anti- hamster conjugated with Alexa Flour 647 (RRID: AB_2535868 Cat#A21451) (Thermo Fisher Scientific Inc., Carlsbad, California, USA).

Quantification of IBA1 immunofluorescence in Foxn1$^{nu/nu}$ brains

Tissue sections from control (n=4) and 28-day metastatic (n=4) Foxn1$^{nu/nu}$ mouse brains were stained for IBA1. Micrographs were acquired on the BZ-X700 Keyence fluorescence microscope. Z-stack micrographs were compressed into maximum intensity projection and opened in FIJI (Fiji, RRID:SCR_002285). Images were quantified for the number of IBA1+ cells in 8-17 fields from 4 control and 4 metastatic mouse brains using the threshold, convert to binary, watershed, and analyze particles functions. Data was tabulated and analyzed in GraphPad Prism 9 (https://www.graphpad.com/scientificsoftware/prism/, GraphPad Prism, RRID:SCR_002798).

CODEX Imaging of Mouse BCBM

C57BL/6 control or EO771-GFP tumor bearing mouse brain tissue was prepared and immunostained following CODEX manual rev. C, and imaged using 20X PlanApo 0.75 NA lens on the CODEX automated imaging system with the Keyence 700 microscope and BZX software. A 7X7 tile scan with 6x Z planes 1.5u steps was taken and processed using the CODEX processor 1.8. The output images were segmented in QuPath using StarDist (https://github.com/stardist/stardist). Segmented cells were phenotyped by a user trained machine learning classifier in QuPath based on the marker expression for TMEM119, IBA1, MHC-II, CD74, ISG15. The following antibodies, barcode/ reporter and fluorescence combinations were used:

TMEM119 (195H4, Synaptic Systems, 1:25) BX/RX035-ATTO550, MHC-II (M5/114.15.2, Akoya) BX/RX001-ATTO550, ISG15 (1H9L21, Thermofisher, 1:40) BX/RX045-CY5, CD74 (In1/CD74, Biolegend 1:40) BX/RX036-CY5, IBA1 (019-19741, Wako 1:80) BX/RX042- CY5. Custom

conjugations of TMEM119, ISG15, CD74 and IBA1 were performed using the Akoya custom conjugation kit and barcodes, following the CODEX manual, and validated by SDS-PAGE and visual assessment of staining compared to standard immunofluorescence with unconjugated antibody and fluorescent secondary antibody on FF mouse spleen or tumor sections.

Isolation of cells for scRNA-seq

Single cell suspensions from mouse brains were prepared using the Adult Brain Dissociation Kit, Mouse and Rat (Miltenyi Biotec) with some modifications. Whole dissected brains were partitioned and placed into C tube (Miltenyi Biotec, Cat#130-093-237) containing enzyme P and A (Foxn1$^{nu/nu}$ mice) or 1mg/mL Collagenase D (Millipore Sigma Cat#11213857001, C57BL/6 and RAG1-KO mice. Brain tissue was digested using gentleMACS Octo Dissociator with heaters operating the 37°C adult brain dissociation protocol. After removal of myelin by density centrifugation, remaining red blood cells were lysed.Cells were blocked with anti-CD16/32 and stained with fluorescent antibodies.. C57BL/6 and RAG1-KO cells from individual mice were labeled using a CellPlex multiplexed oligo labeling kit in accordance with the manufacturer's protocol (10x Genomics Cat# 1000261). The labeled cells were sorted on a BD FACSAria Fusion sorter. For sorting of microglia, astrocytes, cancer cells, and total leukocytes, cells were gated for size based on forward and side scatter, single cells, and Sytox Blue viability (Thermofisher, Cat#S34857. All myeloid cells (CD45$^+$ CD11b$^+$) and astrocytes (CD45$^-$, ACSA2$^+$) were sorted from control and metastatic mouse brains, GFP$^+$ 231BR cells were sorted from metastatic brains, and total leukocytes (CD45$^+$) were sorted from tumor-bearing control and RAG1-KO mouse brains into 500µL of chilled FACS buffer.

scRNA-seq of murine brain leukocytes

FACS isolated mouse immune cells were resuspended in 0.04% BSA in PBS to achieve 1,000 cells/µL. Final cell suspensions were counted on the Countess II automated cell counter.

Cells were loaded onto the 10x Genomics Chromium Single Cell Gene Expression 3' v2 Chemistry kits for GEMs generation. Following the Chromium Single Cell 3′ Reagents Kits version 2 user guide (CG00052 Rev B), cells were loaded to achieve 10,000 cells for capture. Libraries were sequenced on the Illumina HiSeq 4000 (Foxn1$^{nu/nu}$) or NovaSeq 6000 (C57BL/6, RAG1-KO) platform to achieve an average read depth of 50,000 mean reads per cell. Sequencing reads were aligned utilizing 10x Genomics Cell Ranger Count 3.0.2 to a dual indexed GRCh38 and mm10 reference genome.

Flow cytometry analysis of immune cells from control and BCBM mouse brain tissue

Tissue was prepared as for FACS sortingusing 1mg/mL Collagenase D (Milipore Sigma Cat#11213857001) for digestion. Cells were stained with ZombieNIR viability dye (1:500, BioLegend Cat. No. 423106), and blocked with anti-CD16/32 antibody . Next, cells were stained with fluorescent antibodies for extracellular markers and analyzed using BD Fortessa X20 and FlowJo v10 software. For intracellular staining of Foxp3 and CD3e, cells were fixed with the eBioscience Foxp3 /Transcription Factor Staining Buffer set according to the manufacturer's instructions (Thermofisher Cat #00-5523-00) for analysis on the BD Fortessa X20. The following antibodies were used for flow cytometry analysis: CD45-BV510 (Biolegend, 30-F11, 1:100), CD45-FITC (Biolegend, 30-F11, 1:100), CD11b-BV605 (Biolegend, M1/70, 1:200), CD11b-PE (Biolegend, M1/70, 1:200), CD11b-BV650 (Biolegend, M1/70, 1:200), ACSA-2-APC (Miltenyi, REA969, 1:80), Ly6C-BV785 (Biolegend, HK1.41:200, 1:200), Ia/Ie (MHC-II)PacificBlue (Biolegend, M5/114.15.2, 1:500), CD74-AF647 (Biolegend, In1/CD74, 1:100), CD317-PE (Biolegend, 129C1, 1:100), CD3-PerCPCy5.5 (Biolegend, 17A2, 1:100), TCRb-PECy5 (Biolegend, H57-597, 1:100), NK1.1-PEDazzle594 (Biolegend, PK136, 1:100), CD4BV605 (Biolegend, RM4-5, 1:500), CD8a-PacificBlue (Biolegend, 53-6.7, 1:500), Foxp3-PE (eBioscience, FJK-16s, 1:100), CD152 (CTLA-4)-APC (BD, UC10-4F10-11, 1:100), CD44-PECy7

(Biolegend, IM7, 1:100), CD62L-BV785 (Biolegend, MEL-14, 1:100), CD107a-FITC (Biolegend, 1D4B, 1:100).

In vitro differentiation and early postnatal transplantation of iHPCs

Differentiation of Hematopoietic Progenitor Cells from iPSCs (iHPCs) performed according to McQuade et al. (2018). Briefly, iPSCs were first passaged in mTeSR-E8 and transferred to Medium A from the STEMdiff Hematopoietic Kit (Stem Cell Technologies, Cat#05310). On day three, flattened endothelial cell colonies were transferred to Medium B for seven days. On day 10, non-adherent $CD43^+$ iHPCs were collected and frozen in Bambanker (Fisher Scientific, Cat#NC9582225) for later transplantation. Cells were thawed in iPS-Microglia medium (DMEM/F12, 2X insulin-transferrin-selenite, 2X B27, 0.5X N2, 1X glutamax, 1X non-essential amino acids, 400 mM monothioglycerol, and 5 mg/mL human insulin freshly supplemented with 100ng/mL IL-34,
50ng/mL TGFb1, and 25 ng/mL M-CSF (Peprotech, Cat#100-21) according to McQuade et al, 2018[52]. Early Postnatal Intracerebroventricular Transplantation of iHPCs was performed as described in Hasselmann et al, 2019[53].

Isolation of human xenotransplanted microglia

10-week-old, MITRG mice were injected intracardially with 500,000 mCherry labeled 231BR cells as previously described. 25 days post-injection, following perfusion with ice cold PBS containing 5µg/ml actinomycin D (act D, Cat#A1410), whole metastatic brains were imaged on a dissection microscope (Leica Biosystems, DMC 2900) for mCherry and GFP intensity. Half brains were dissected, fixing the left hemisphere in 4% PFA for histology. The right hemisphere was prepped for dissociation as described in Hasselmann et al, 2019[53] with modifications. The cerebellum was removed and the whole right hemisphere was stored briefly in RPMI 1640 containing 5µg/mL act

D, 10μM triptolide (Sigma-Aldrich, Cat#T3652), and 27.1ug/mL anisomycin (Sigma-Aldrich, Cat#A9789). Tissue dissociation was performed using the Tumor Dissociation kit, human (Miltenyi Biotec) as previously described with the kit's enzymes, 5μg/mL act D, 10μM triptolide, and 27.1ug/mL anisomycin using the preprogrammed soft tumor protocol.. Myelin and debris removal was performed in 8mL 23% Percoll (GE Healthcare, Cat#45-001-748), overlaid with 2mL of 1X DPBS.

MULTI-seq labeling and scRNA-seq of human microglia

Individual mice were barcoded following the "MULTI-seq lipid- tagged indices for sample multiplexing for scRNAseq" protocol[64]. Lipid anchor and co-anchor reagents were a gift from Zev Gartner, and barcode index oligos were purchased from Integrated DNA Technologies, Inc. Mouse cell removal was performed using mouse cell removal beads (Miltenyi Biotec)separated using LS columns and the MidiMACs separator (Miltenyi Biotec).Control and metastatic samples were then pooled separately. Cells were resuspended to ~1,000 cells per microliter in FACS buffer, according to counts performed on a hemocytometer.

ScRNA-seq of MITRG human microglia

Final cell suspensions were counted on the Countess II automated cell counter to determine actual concentration for droplet generation. Cells were loaded onto the 10x Genomics Chromium Single Cell Gene Expression 3' v3 Chemistry kits for GEMs generation. Following the Chromium Single Cell 3′ Reagents Kits version 3 user guide (CG000183 Rev C), cells were loaded to achieve 10,000 cells for capture. MULTI-seq barcode libraries were prepared according to the MULTI-seq protocol[62]. Libraries were sequenced on the Illumina NovaSeq 6000 platform to achieve an average read depth of 50,000 mean reads per cell for 3' gene expression libraries.
MULTI-seq barcode libraries were sequenced to achieve at least 5,000 reads per cell.

Sequencing reads were aligned utilizing 10x Genomics Cell Ranger Count 3.1.0 to a dual indexed GRCh38 and mm10 reference genome. All libraries were aggregated using 10x Genomics Cell Ranger Aggr 3.1.0, to normalize the number of mean reads per cells. MULTI-seq reads were processed according to the MULTI-seq protocol (https://github.com/chris-mcginnis-ucsf/MULTIseq).

Analysis of BCBM in FIRE mice

Four six-week-old *Csf1r*$^{\Delta FIRE/\Delta FIRE}$ (FIRE-KO) and *Csf1r*$^{FIRE/FIRE}$ (FIRE-WT) mice were injected intracranially in the right coronal suture with 100,000 enhanced GFP and luciferase labeled EO771 cells as previously described. Mice were imaged for luciferase luminescence one day after injection, and every three days thereafter until endpoint. Imaged mice were anesthetized via isoflurane inhalant and administered 300µg D-Luciferin (Goldbio), intraperitoneally, in sterile DPBS. Following a 10-minute incubation, mice were imaged for bioluminescence utilizing an IVIS Lumina III In Vivo Imaging System (Xenogen). Regions of interest were selected around each brain and average photon flux (total photons/s-cm^2) was recorded using Living Image analysis software (**RRID:SCR_014247,** http://www.perkinelmer.com/catalog/category/id/living%20image%20software) with average background flux subtracted. At endpoint, mice were weighed, euthanized, and dissected and whole brains were removed and placed in a 24 well tissue culture plate submerged in ice cold PBS with D-Luciferin (1.5 mg/mL, Goldbio, Cat# LUCK-1G). After 10 minutes incubation, whole brains were placed on a black plastic card and imaged for luminescence for 1 second. A region of interest was drawn around each brain and the total flux (ptotal photons/s-cm^2) was recorded for analysis.

Fingolimod (FTY720) HCL dosing

FTY720 was purchased from Selleckchem (Cat. no. S5002), and reconstituted to a final concentration of 1mg/mL FTY720 (0.001% DMSO). Mice were injected intraperitoneally with 50-100uL of vehicle or 5mg/kg FTY720 immediately before intracranial injection with 70,000 EO771 eGFP eFFly cells, and daily for the duration of the experiment. The experimental groups were blinded when performing intracranial injections of EO771 cells.

Cytokine screen of Microglia

Control and 14-day EO771 eGFP eFfly tumor bearing brains were digested into single cell suspension as previously described for flow cytometry. Cells were immunostained for BV510-CD45 (Biolegend clone 30-F11, 1:100), BV605-CD11b (Biolegend clone M1/70, 1:200), BV785-Ly6C (Biolegend clone HK1.4, 1:200), and PE/Dazzle594-NK1.1 (Biolegend clone PK136, 1:100). 50,000 microglia per sample were FACS sorted based on CD45lo, CD11b+ Ly6C- NK1.1- expression into FACS buffer. Cells were resuspended in 100uL of 150mM sodium chloride, 1%NP-40 50mM Tris pH 8.0 cell lysis buffer containing 1X Halt Protease Inhibitor (Thermo Fisher Cat. #78430) and stored frozen at -80°C. Frozen samples were shipped on dry ice to Eve Technologies Corp (3415A - 3 Ave.,
NW, Calgary, AB T2N 0M4) to perform a standardized mouse cytokine array / chemokine array 31-Plex (MD31, Millipore MILLIPLEX). The analytes tested for include Eotaxin, G-CSF, GM-CSF, IFNgamma, IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1alpha, MIP-1beta, MIP-2, RANTES, TNFalpha, VEGF.

Human/mouse cell assignment

Cells were aligned to a merged GRCh38/mm10 genome using Cell Ranger v3. Cell species was determined based on the frequency of reads aligning to the mouse genome with very low-quality cells with <200 genes (nFeature_RNA) filtered before estimating. Cells were called as mouse for all cells above the top elbow in the mouse read mapping frequency plot (>0.875 for Foxn1$^{nu/nu}$ data; >0.95 for MITRG data), human for all cells below the bottom elbow (<0.05 for Foxn1$^{nu/nu}$ data; <0.1 for MITRG data), and any other cells were discarded as doublets or poor quality. Any counts for GRCh38 genes in the cells called as mouse were removed from the expression matrix and vice versa for mm10 genes in human cells.

Quality control metrics

Cells for the Foxn1$^{nu/nu}$ cell type identification analysis were filtered to have between 500 and 2000 genes (nFeature_RNA) and <10% mitochondrial genome reads (percent.mito) in any retained cell. Cutoffs were selected based on analysis of violin plots for visual outliers. Putative microglia/astrocyte doublet clusters with marker gene co-expression wereremoved from the Foxn1$^{nu/nu}$ microenvironment. This cell set was then used for subset myeloid and astrocyte analyses based on the cell type labels. Cells were filtered for the myeloid analysis to have <5% percent.mito and low ribosomal expression (<10% of their transcriptome representing Rps and Rpl genes). Cells for the MITRG analysis were filtered to have <20% percent.mito. Doublets and empty gems (Negative) were removed from the MITRG analysis based on MULTI-Seq barcoding label assignment from the R package deMULTIplex. Cell cycle signatures (S.Score and G2M.Score, determined by CellCycleScoring in Seurat) were regressed from the data for the 231BR analysis as well as the MITRG analysis before clustering and dimensionality reduction.

Clustering and differential expression

Main clustering and dimensionality reductions were performed in Seurat using the default Louvain and tSNE methods. UMAP was used for dimensionality reductions in microglia subclustering analyses to better visualize global relationships. Some datasets were integrated using the mutual kNN algorithm adaptation in Seurat before these steps. Specifically, integration was performed on the Foxn1$^{nu/nu}$ full microenvironment and astrocyte analyses by sequencing batch (Con1:Met1, Con2:Con3, Met2:Met3). Integrated analyses used the “vst” selection method with nfeatures=2000 for FindVariableFeatures and dims=1:30 for FindIntegrationAnchors and IntegrateData. Differential expression analyses were run on the RNA assay in Seurat with FindAllMarkers/FindMarkers using the Wilcoxon rank sum test and adjusted *P* values represent the Bonferroni corrected values for all single-cell analyses. Cell types and states were assigned to clusters manually based on gene expression profiles. All plotting functions through Seurat utilize ggplot2.

GO term analysis and gene scoring

GO term analyses were performed using the MouseMine[32] web portal with list input for M. musculus with the default background population for mouse analyses and using the Enrichr portal[65,66] with a gene list input. Gene inputs included only genes considered differentially expressed with a Bonferroni adjusted *P* value < 0.05 from the Wilcoxon rank sum test.GO terms were selected from the Gene Ontology Enrichment section for biological_process with Holm-Bonferroni adjusted *P* value < 0.05 in MouseMine or the GO Biological Process 2018 list in Enrichr with unadjusted *P* value < 0.05. All gene scoring on singlecell data was performed in Seurat using the AddModuleScore function with default parameters.

MG score gene list was taken directly as the Core MG list from Supplementary Table 3 in Bowman et al, 2016[30].

Topic scores were determined for the MITRG mouse using the top 25 marker genes of each topic (ExtractTopFeatures with method = "poisson", options="min", and shared = FALSE), translated to human using the biomaRt package.

Latent Dirichlet Allocation, Topic model

To fit a topic model using Latent Dirichlet Allocation (LDA), we used the R package 'CountClust'[33] which was optimized for use on RNA-seq datasets. As input to our model, we provided a raw counts matrix containing all cells labeled as microglia and all detected genes from our Foxn1$^{nu/nu}$ dataset. The topic model was fit using the 'FitGoM' function, with a range of cluster numbers (K), and an error tol = 10. We chose the model with K = 15 since it achieved a relatively low value for the Bayesian Information Criterion (BIC) and had enough resolution to provide topics with unique, biologically interpretable gene lists. Top gene markers for each topic were identified using the function 'ExtractTopFeatures' with method='poisson', options='min' and shared=TRUE for the marker heatmap (or shared = FALSE for gene scores).

Pseudotemporal Ordering of Cells

Monocle 3[67] was used for trajectory inference and pseudotemporal ordering of cells. For input, the final annotated Seurat object was converted to a Monocle 3 cds object using the SeuratWrappers function as.cell_data_set. The counts data matrix was then processed with the standard Monocle 3 pipeline using default parameters in the preprocess_cds and reduce_dimension functions. Clustering was performed with cluster_cells using resolution=3e-4 to maintain similar cluster assignments between Seurat and Monocle 3. The principal graph was constructed with the learn_graph function using one partition. To identify the root principal point for ordering cells, the helper function get_earliest_principal_node was used as defined in the Monocle 3 vignette, using D4_BL6 cells in our timepoint_strain metadata column.

Pseudotemporal ordering was then performed using order_cells with root_pr_nodes=get_earliest_principal_node(cds). To display the pseudotime data on the original Seurat UMAP embeddings, the Monocle cds object was converted to a Seurat object with the Seurat function as.Seurat and the pseudotime metadata column of the resulting object was transferred to the original Seurat object using AddMetaData. The pseudotime results were displayed as a feature plot using the plasma color palette from the viridis library.

Survival analysis

Survival analysis was performed using the Brain-Met samples from Vareslija et al, 2018[55], based on the column header “M_” from their Github uploaded counts matrix (https://github.com/npriedig/jnci_2018/blob/master/brainMetPairs.salmon.cts.txt). This subset of the counts matrix was converted to log(cpm + 1) using the ‘cpm’ function in edgeR. This matrix was then merged with the clinical information from Table 1 of Vareslija et al, 2018[55], resulting in 20 total samples (samples "7M_RCS" and "19.2M_Pitt" were dropped, the first due to a lack of matching clinical data and the second due to sample replication). Our genes and signatures of interest, CD74, BST2, MHC-II genes ("HLA-DMA", "HLA-DMB", "HLA-DOA", "HLA-DOB", "HLADPA1", "HLA-DPB1", "HLA-DQA1", "HLA-DQA2", "HLA-DQB1", "HLA-DQB2", "HLA-DRA", "HLA-DRB1", "HLA-DRB3", "HLA-DRB4", "HLA-DRB5") and microglia ("P2RY12", "TMEM119", "GPR34", "CX3CR1", "CD81", "SELPLG") were converted to their Ensembl IDs using ‘mapIds’ with multiVals = ‘list’ from org.Hs.eg.db, and added to the dataset as log(cpm +1) for single genes, and the sum of the scaled data (z-scores) for multigene signatures. Survival analysis was performed using the R package survminer, wheredata stratification was made using ‘surv_cutpoint’ and ‘surv_categorize’ to identify an optimal split, and the KM curves generated using ‘survfit’ in ‘ggsurvplot’.

Statistics and Reproducibility

No statistical methods were used to pre-determine sample sizes but sample sizes are similar to those reported in previous publications. Data distribution was assumed to be normal but this was not formally tested. Mice with insufficient viable cell yield were excluded from analysis by flow cytometry. Where possible, experimental groups were randomized prior to initiation of experiments. Investigators were not blinded to allocation and experimental outcome except where otherwise indicated.

Data Availability

RNA-seq data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession codes GSE147949 and GSE237386. Reference genome GRCh38/mm10 are available from Ensembl. MULTI-seq reads were processed according to the MULTI-seq protocol[62] and available on GitHub (https://github.com/chris-mcginnis-ucsf/MULTIseq). Qptiff images were segmented in QuPath using StarDist and is available on GitHub (https://github.com/stardist/stardist)." All other data are available from the corresponding author on reasonable request.

Methods-only References

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