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

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1 Editor summary:

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

5

6

7 Reviewer Recognition:

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

Figure or Table #	Figure/Table title	Filename	Figure/Table Legend
Please group Extended Data items by type, in sequential order. Total number of items (Figs. + Tables) must not exceed 10.	One sentence only	Whole original file name including extension. i.e.: Smith_ED_Fig1.jpg	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	<p>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.</p> <p>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.</p>

			<p>Results are representative of a single experiment.</p> <p>Scale bar = 50 μm.</p> <p>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.</p> <p>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.</p> <p>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).</p>
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			<p>f. Bar chart shows the frequency of cells contributed by each mouse that localize to each cell type in Figure 1f.</p> <p>g. tSNE plot shows astrocytes colored by control or metastatic condition.</p> <p>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 $-\log_{10}$ of Bonferroni corrected P values, and the x-axis represents average natural logFC between conditions.</p>
Extended Data Fig. 2	Identification of myeloid cell types and subclustering analysis of	ED_Fig2.pdf	<p>a. tSNE plot shows myeloid cells (n=15,288) colored and labeled by cell type. mDC = mature dendritic cell. Mono.Macro = monocytes and macrophages.</p>

	proinflammatory microglia		<p>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.</p> <p>c. Bar chart shows the frequency of cells contributed by each mouse that localize to each cell type in b.</p> <p>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).</p> <p>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.</p> <p>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</p>
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			<p>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. $\min(\text{Metastatic}) > \max(\text{Control})$).</p>
Extended Data Fig. 3	Pro-inflammatory marker expression in microglia from BCBM models	ED_Fig3.pdf	<p>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.</p> <p>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. <i>P</i> values were generated by an unpaired two-</p>

			<p>sided student's <i>t</i>-test, and error bars indicate mean +/- standard deviation.</p> <p>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-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-II⁺), distal < 0.01, tumor = 0.41. Scale bar= 100um.</p>
Extended Data Fig. 4	Quantification of tumor size in FIRE-	ED_Fig4.pdf	<p>a. IVIS images show EO771 luciferase luminescence signal change over time in FIRE-WT and FIRE-KO animals. Representative animals that displayed</p>

	WT and FIRE-KO animals		<p>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/cm²/sr).</p> <p>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 (<10⁶) or >5-fold decrease in signal relative to maximum.</p> <p>c. Serial dilution analysis of EO771 cell engraftment in FIRE-WT and FIRE-KO animals. 10-200 x 10⁴ 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.</p>
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			<p>Fractions denote the number of grafts that produced macroscopic tumors in each condition.</p> <p>d. Dot plots quantify luminescent signal (total flux) from each tissue shown in Extended Data Figure 4c at day 14. <i>P</i> values were generated by an unpaired two-sided student's <i>t</i>-test.</p>
Extended Data Fig. 5	Analysis of NK, T, and monocyte responses to BCBM in FIRE-WT and FIRE-KO animals	ED_Fig5.pdf	<p>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/cm²/sr), and bar graph shows quantification of total flux (p/s). <i>P</i> value shown is the result of a student's unpaired two sided <i>t</i>-test. Error bars represent mean +/- standard deviation.</p> <p>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</p>

			<p>whiskers are indicative of the first through fourth interquartile range. <i>P</i> value shown is the result of a student's un-paired two sided <i>t</i>-test.</p> <p>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.</p> <p>d. Quantification of monocytes in tumor bearing FIRE-WT and FIRE-KO brain tissues by flow cytometry. CD11b⁺Ly6C⁺ monocytes were identified following</p>
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			<p>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.</p> <p>e. Linear regression model of CD8⁺ T cell and Treg quantification from Extended Data Figure 5b. R-squared and <i>P</i> values determined by simple linear regression function.</p>
Extended Data Fig. 6	Analysis of tumor burden and the immune response in T cell deficient mice	ED_Fig6.pdf	<p>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.</p> <p>b. Gating scheme for analysis of microglia and monocytes in brain tissue from vehicle, FTY720 and RAG1-KO animals. FACS plots (top panels) show</p>

			<p>gating for CD45^{hi-int} and CD11b⁺ cell populations, followed by gating for CD45^{hi}Ly6C⁺ monocytes and CD45^{int}Ly6C⁻ microglia (bottom panels).</p> <p>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.</p> <p>d. Quantification of EO771 tumor burden at endpoint on day 12 by IVIS. Pseudocolor shows radiance (p/sec/cm²/sr) in each whole brain.</p>
Extended Data Fig. 7	Analysis of BCBM immune repertoire in T cell deficient and replete mice	ED_Fig7.pdf	<p>a. Dot plot shows top marker genes for each cell type in total CD45^{hi-int} sorted cells, ranked by the average log₂ 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</p>

			<p>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.</p> <p>b. UMAPs show T cells (n=1949 cells) from C57BL/6 mice at day 4 and 10, colored by cluster (left) and timepoint (right).</p> <p>c. Bar graph illustrates the distribution of T cell clusters in each animal (n=6) separated by timepoint.</p> <p>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, $\gamma\delta$ = gamma delta T cell.</p> <p>e. Dot plot shows expression of top marker genes for each microglia cell cluster from Figure 7b.</p>
Extended Data Fig. 8	Analysis of humanized mouse	ED_Fig8.pdf	<p>a. Whole mount brightfield and fluorescence microscopy images show brains from MITRG mice transplanted</p>

	model of BCBM and patient BCBM data		<p>with GFP-labeled iHPSC cells and mCherry-labeled 231BR cells from Figure 8a.</p> <p>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.</p> <p>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).</p> <p>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.</p>
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			<p>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.</p> <p>f. Bar chart shows the frequency of cells contributed by each mouse to the cell types shown in e.</p> <p>g. tSNE plots colored to show the expression of BST2, CD74 and CCL3.</p> <p>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</p>
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			<p>weights. Scaling was performed by dividing all topic scores by the maximum topic score across the dataset.</p> <p>i. Bar plot shows the relative enrichment of each topic score in human microglia.</p> <p>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).</p>
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Item	Present?	Filename	A brief, numerical description of file contents.
		Whole original file name including extension. i.e.: Smith_SI.pdf. The extension must be .pdf	i.e.: <i>Supplementary Figures 1-4, Supplementary Discussion, and Supplementary Tables 1-4.</i>
Supplementary Information	Yes	FACS gating strategies.pdf	FACS gating strategies
Reporting Summary	Yes	[F] NCB-L45274D	

Peer Review Information	Yes	<i>Peer Review file.pdf</i>
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Type	Number	Filename	Legend or Descriptive Caption
	<p>Each type of file (Table, Video, etc.) should be numbered from 1 onwards.</p> <p>Multiple files of the same type should be listed in sequence, i.e.: Supplementary Video 1, Supplementary Video 2, etc.</p>	<p>Whole original file name including extension. i.e.: <i>Smith_Supplementary_Video_1.mov</i></p>	<p>Describe the contents of the file</p>
Supplementary Table	Supplementary tables 1.xlsx	Supplementary tables	Supplementary tables 1-6

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Parent Figure or Table	Filename	Data description
	<p>Whole original file name including extension. i.e.: <i>Smith_SourceData_Fig1.xls</i>, or <i>Smith_Unmodified_Gels_Fig1.pdf</i></p>	<p>i.e.: Unprocessed western Blots and/or gels, Statistical Source Data, etc.</p>
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

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

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51

52 **Keywords**

53 Breast cancer, brain metastasis, single cell, RNA sequencing, microglia, macrophages, tumor
54 associated macrophage, TAM, FIRE, *Csf1r*^{ΔFIRE/ΔFIRE}

55
56 **Abstract**

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

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78 **Introduction**

79 Breast cancer brain metastasis (BCBM) is rapidly emerging as a critical problem in breast
80 cancer. 15-30% of metastatic breast cancer patients develop brain metastasis, and studies project
81 a >30% increase in patients as treatments for peripheral disease improve and patients live
82 longer^{1,2}. This is alarming since there are no effective treatments and median survival is only a
83 few months³⁻⁶. There is growing interest in immunotherapeutic strategies to treat central nervous
84 system (CNS) cancers, given that immune cells enter the brain while most conventional therapies
85 are excluded by the blood brain barrier^{7,8}. Greater understanding of the immune response to
86 BCBM will be needed to develop immunotherapy strategies effective in the unique immune
87 microenvironment of the CNS.

88 The brain immune microenvironment is principally composed of specialized tissue
89 resident macrophages called microglia that tile the brain and play diverse functions in CNS
90 homeostasis and disease⁹⁻¹¹. Microglia represent an attractive immunotherapeutic target because
91 they are the first line of defense to disease in the CNS. BCBMs are heavily infiltrated with tumor
92 associated macrophages (TAMs), which may be comprised of microglia, macrophages and bone
93 marrow derived myeloid cells¹²⁻¹⁶. Functional studies suggest a tumor promoting role for TAMs.
94 Depletion of TAMs with CSF1R inhibitors results in tumor reduction and decreased metastasis in
95 glioblastoma and melanoma models¹⁷⁻²¹. TAM depletion using a CX3CR1-targeted genetic
96 ablation model similarly results in decreased BCBM²². However, it is unclear whether microglia or
97 other TAMs produce these tumor promoting effects. CSF1R inhibitors deplete microglia but also
98 attenuate other myeloid cells, and microglia ultimately repopulate the brain when treatment
99 ceases. Likewise, CX3CR1 is expressed by diverse myeloid cell populations and upregulated by
100 myeloid cells upon entry into the brain²³. Therefore, the specific impact of microglia on tumor
101 initiation and their potential as an immunotherapy target remain unclear.

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

104 responses. ScRNA-seq of >90,000 cells from three different BCBM models revealed that
105 microglia mount a robust pro-inflammatory response to BCBM. Using a genetic knockout model,
106 we find that animals lacking microglia show increased metastasis, decreased survival, and
107 impaired NK and T cell responses to BCBM²⁴. We show that ablation of T cells reduces microglia
108 activation and attenuates their tumor suppressive effect, indicating that reciprocal microglia-T cell
109 activation is critical to suppress BCBM. Utilizing a humanized mouse model, we find that the pro-
110 inflammatory response is conserved in human microglia, and analysis of patient BCBM data
111 indicates that increased pro-inflammatory marker expression is associated with better prognosis.
112 These findings contrast with the pro-tumorigenic function reported for TAMs and highlight the
113 potential of harnessing the anti-tumor function of microglia to treat brain metastasis.

114

115 Results

116 Single-cell analysis of TAMs in BCBM

117 We used scRNA-seq to interrogate the microglia response to BCBM using the MDA-MB-
118 231-BR (231BR) model. In this model, GFP-labeled 231BR cells are delivered into the arterial
119 circulation via intracardiac injection and form parenchymal brain metastases by day 28 (**Fig 1a,b,**
120 **Extended Data Figure 1a**)^{26,27,28,29}. Like in human BCBM, metastases are heavily infiltrated with
121 IBA1+ (ionized calcium-binding adaptor molecule 1) TAMs (**Fig 1b,c, Extended Data Figure**
122 **1b**).²⁵ For scRNA-seq, cells were dissociated from control and metastatic brains and myeloid cells
123 were isolated by flow cytometry (**Fig 1d, Extended Data Figure 1c**). Cancer cells and astrocytes
124 were sorted for control (**Extended Data Figure 1c, Fig 1d**). Analysis of the 42,891 cells that
125 passed quality control filtering (**Extended Data Figure 1d,e**) revealed seven distinct cell types
126 identified by canonical markers (**Fig 1e,f, Supplementary Table 1**). This included the targeted
127 cell types, astrocytes (*Aldoc*, *Atp1a2*), microglia (*Tmem119*, *P2ry12*) and non-microglia myeloid
128 cells (*Lyz2*, *Plac8*) (**Fig 1e,f, Supplementary Table 1**). We also recovered small numbers of
129 ependymal cells (*Ccdc153*, *Rarres2*), oligodendrocytes (*Mbp*, *Ptgds*), vascular cells (*Cldn5*, *Vtn*),

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

135

136 Microglia display robust pro-inflammatory responses to BCBM

137 In contrast to astrocytes, analysis of the myeloid cells revealed strong separation of control
138 and metastatic conditions (Fig 2a). Microglia were distinguished from other myeloid populations
139 by scoring each cell for the core microglia signature developed by Bowman et al (2016) (Fig 2b,
140 Supplementary Table 3)³⁰. This identified two large microglia populations (*Tmem119*, *P2ry12*,
141 *Sparc*, *Gpr34*), where one contained microglia from both control and metastatic and the other was
142 almost entirely from metastatic (Fig 2a,b, Extended Data Figure 2a-d). We also found two small
143 populations of microglia that display an increased stress response (Extended Data Figure 2d),
144 which is common post tissue manipulation³¹. Neutrophils (*Camp*, *S100a9*),
145 monocytes/macrophages (*Ly6c2*, *Lyz2*), mature dendritic cells (*Ccr7*, *Flt3*), and B cells (*Igkc*,
146 *Cd79a*) were also identified (Fig 2b, Extended Data Figure 2a-d, Supplementary Table 4).

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

156 overlapping gene modules are expressed in a population of cells³³. This analysis identified four
157 core topics (**Extended Data Figure 2e-f, Supplementary Table 5**). Topic 12 was the most
158 broadly upregulated and represented an interferon (IFN) response program (*Bst2, Ifitm3, Ifit3b,*
159 *Isg15*), which has been previously reported by microglia in other disease contexts^{34, 35, 36} (**Fig 2e-**
160 **g**). This likely represents the initial sensing of microglia to metastatic infiltration and tissue
161 damage³⁷. Topic 15 showed a more restricted expression pattern and was enriched for genes
162 associated with antigen presentation (AP) (*Cd74, H2-Aa, H2-D1*) (**Fig 2e-g**), which has also been
163 observed in glioma and Alzheimer's Disease (AD)^{34,36}. AP genes enable antigen presentation to
164 T cells, raising the question of whether microglia present antigen to T cells in the CNS. Topic 14
165 was expressed by a small subset of microglia and was associated with a secretory phenotype
166 (**Fig 2e-g**). This topic was enriched for genes associated with exosomes (*Cd63*), lipid metabolism
167 (*ApoE, Lpl*), and cytokines (*Spp1, Csf1, Il1b, Tnf*) (**Fig 2e,g**). This topic strongly overlaps with the
168 signature of disease-associated microglia or "DAMs", a population of phagocytic microglia
169 identified in neurodegeneration³⁸. The IFN response and AP topics both included genes encoding
170 numerous chemokines for immune cell trafficking (**Fig 2e,g, Supplementary Table 5**). A final
171 topic (topic 3) was enriched for ribosomal genes (**Extended Data Figure 2f, Supplementary**
172 **Table 5**), which could indicate cells with increased transcriptional capacity or stress response.
173 These data show that microglia upregulate multiple pro-inflammatory programs, suggesting they
174 play diverse roles in the immune response to BCBM.

175

176 **The microglia response is conserved in diverse BCBM models**

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

182 We used a multiplex IF system (co-detection by indexing, CODEX) for in situ validation.
183 We co-stained for MHC-II, CD74, and IFN-stimulated gene 15 (ISG15), as well as TMEM119 and
184 GFP to identify microglia and tumor cells, respectively. We found that pro-inflammatory microglia
185 localize proximal to tumor cells, while distal microglia are negative **(Fig 3b,c, Extended Data**
186 **Figure 3c)**. The highest frequency of microglia co-expressed all three markers (MHC-
187 II⁺CD74⁺ISG15⁺, 29%) **(Fig 3c)**. We also observed subpopulations of microglia that express only
188 the AP markers (MHC-II⁺CD74⁺ISG15⁻, 11%) or the IFN response marker (MHC-II⁻CD74⁻ISG15⁺,
189 11%) **(Fig 3c)**. These data are consistent with our topic modeling, showing substantial marker
190 overlap but notable exclusivity of the AP and IFN response programs into different subsets of
191 microglia.

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

198

199 **Animals lacking microglia show increased tumor progression**

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

208 were injected with GFP and luciferase-labeled EO771 cells and monitored by *in vivo*
209 bioluminescence (IVIS) (Fig 4c). Surprisingly, many FIRE-KO mice quickly developed overt
210 clinical symptoms of advanced disease (Fig 4d,e). Five of 14 FIRE-KO mice died before endpoint
211 (36% mortality), while all 19 FIRE-WT survived (0% mortality) (Fig 4d). Surviving FIRE-KO mice
212 also displayed >20% decrease in body mass compared to FIRE-WT, indicating increased
213 morbidity (Fig 4e). IVIS imaging revealed differences in the kinetics of tumor growth over time
214 (Extended Data Figure 4a,b). We observed tumor rejection in eight of 19 FIRE-WT mice, while
215 signal continued to increase in all 14 FIRE-KO animals (Fig 4f, Extended Data Figure 4a,b). We
216 further compared tumor engraftment in FIRE-KO and FIRE-WT mice using a serial dilution
217 approach. This showed increased engraftment efficiency and larger tumor growth in FIRE-KO
218 compared to FIRE-WT mice (Extended Data Figure 4c,d). Together, these data show that
219 animals lacking microglia demonstrate increased tumor growth and engraftment, and decreased
220 capacity for tumor rejection.

221

222 **Microglia promote NK and T cell responses to BCBM**

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

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

244

245 **Microglia and T cells coordinate the anti-tumor response**

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

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

260 percentages of the AP markers MHC-II+ ($p=0.0400$) and CD74+ ($p=0.0271$), respectively (**Fig**
261 **6e**). The reduced expression of these proteins was even more pronounced in RAG1-KO mice
262 (**Fig 6e**), indicating that this is not simply an effect of FTY720 treatment. Furthermore, FTY720
263 treated and RAG1-KO mice had a 2-fold ($p=0.0160$) and 9.4-fold ($p<0.0001$) higher percentage
264 of microglia positive for the IFN response protein BST2, respectively (**Fig 6e**). This suggests that
265 T cells may be required to fully license microglia to upregulate the AP program, and without T
266 cells, microglia are limited to the IFN response program.

267

268 **Altered microglia activation in animals lacking T cells**

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

280 We performed subset analysis of microglia to determine how their gene expression
281 changes over time in parallel with T cell activation (**Fig 7b, Extended Data Figure 7e**).
282 Subclustering of microglia showed similar populations as we previously identified (**Fig 7b**). We
283 scored each microglia in the dataset for expression of top genes associated with each topic (**Fig**
284 **7c, Supplementary Table 3**), IFN response, secretory, and AP. This showed that all three
285 programs increase over time from day 4 to 10 in C57BL/6 animals. At day 4, we observed the

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

303

304 **The pro-inflammatory response is conserved in human microglia**

305 We investigated the pro-inflammatory response in human microglia and its relevance in
306 BCBM patients. We developed a humanized mouse model of BCBM based on prior work, where
307 MITRG mice (human *CSF1*, *IL3* and *TPO* knock in to *Rag2^{-/-}Il2ry^{-/-}* mice) are reconstituted with
308 human microglia and macrophages following transplantation of human induced pluripotency-
309 derived hematopoietic progenitor cells (iHPSCs) into the postnatal brain⁵²⁻⁵⁴. In contrast to patient
310 BCBM samples, we were able to use these animals to investigate the initial response of human
311 microglia to tumor initiation. We injected MITRG mouse pups with GFP-labeled iHPSCs, allowed

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

317 Clustering and marker gene analysis revealed a distinct population of 231BR cells (*VIM*)
318 and several populations of myeloid cells **(Fig 8b, Extended Data Figure 8b-f, Supplementary**
319 **Table 6)**. These included human perivascular macrophages (*CD163*), microglia (*TMEM119*,
320 *P2RY12*), and a population of proliferating myeloid cells (*MKI67*) **(Fig8b, Extended Data Figure**
321 **8d-f, Supplementary Table 6)**. We identified 4,904 genes differentially expressed between
322 microglia from control and metastatic brains **(Supplementary Table 6)**. Gene Ontology (GO)
323 analysis revealed that similar pathways were upregulated in human microglia as observed in
324 mouse **(Fig 8c)**. We used gene scoring to investigate human microglia heterogeneity and
325 expression of the core topics upregulated in mouse BCBM **(Fig 2d-f, Supplementary Table 3)**.
326 This showed distinct but overlapping expression of the IFN response, AP, and secretory programs
327 in subsets of human microglia like observed in mouse **(Extended Data Figure 8g-i)**. Importantly,
328 upregulation of the IFN response and AP topics in this model was not as robust as observed in
329 mouse microglia **(Extended Data Figure 8i)**. This is consistent with the more severe immune
330 defects in MITRG mice and our findings that T cells are important for microglia activation.

331 We compared the prognostic relevance of microglia signatures in BCBM patients using a
332 bulk RNA sequencing dataset of human BCBM tumors⁵⁵. We found that patients with a high
333 expression of canonical microglia markers had significantly better overall survival, suggesting
334 increased microglia infiltration is associated with better outcomes **(Fig 8d)**. We further found that
335 higher expression of key genes characteristic of the AP (MHC-II) and secretory programs (CSF1)
336 are associated with increased overall survival, while higher expression of the IFN response gene
337 BST2 is associated with decreased survival **(Extended Data Figure 8j)**. These data suggest that

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

343

344 Discussion

345

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

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

364 has been observed in other depletion models^{59,60}. The massive cell death produced in the
365 Cx3cr1^{CreERT/+};Rosa26^{iDTR/+} depletion model has also been shown to induce cytokine storm and
366 astrogliosis, which may have confounding effects on tumor growth and the immune response^{61,62}.
367 Another important distinction is that the FIRE-KO mice lack microglia from birth, while most prior
368 studies targeted TAMs postnatally and after tumor initiation. It is therefore plausible that the timing
369 of depletion impacts the outcome, as microglia and TAMs may become tumor-promoting as
370 disease progresses.

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

378

379

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407

408 **Author contributions**

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

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

414

415 **Competing interests statement**

416 The authors declare no competing interests.

417

418 **Figure legends**

419 **Figure 1: Single-cell analysis of TAMs in BCBM**

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

425 b. IF staining shows IBA1⁺ cells (red) in control and metastatic brains at 7, 14 and 28 days post
426 231BR cell injection. Metastatic cells are GFP⁺ (green). 231BR cells arrest in blood vessels
427 and cross into the brain 2-7 days post-injection, then grow along blood vessels forming
428 micrometastases by day 14, parenchymal metastases by day 28. Scale bar = 50 μ m.

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

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

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

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

445

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

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

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

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

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

- 466 e. Heatmap shows three core topics upregulated in microglia in BCBM. Scaled gene weights
467 for top genes comprising each topic are shown.
- 468 f. tSNE plots show distribution of three core topics in each microglia. Left panels show topic
469 weight in each cell indicated by contrast gray scale. Right panel overlay shows top topic
470 assignment for each cell, where only cells with a topic weight > 0.1 are colored.
- 471 g. tSNE plots show expression of selected genes from each topic in myeloid cells.

472

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

- 474 a. Flow cytometry analysis of CD74, BST2 and MHC-II expression in microglia from four BCBM
475 models. Microglia were identified by gating on CD45^{lo}, CD11b⁺, Ly6C⁻ cells. Bar graphs show
476 the percent of microglia expressing each marker. *P* values were generated by an unpaired
477 two-sided student's *t*-test, error bars indicate mean +/- standard deviation.
- 478 b. In situ analysis of microglia pro-inflammatory marker expression by multiplex IF (CODEX).
479 Brain tissue slices from mice bearing EO771-GFP tumors were stained for DAPI (blue), and
480 antibodies against GFP (green), TMEM119 (red), MHC-II (white), CD74 (yellow), and ISG15
481 (cyan). Left panel shows overview of all markers. Scale bar = 840 microns. Right panels show
482 pairwise marker expression in higher magnification insets of tumor and distal regions. Short
483 arrows indicate representative microglia expressing AP markers (TMEM119⁺MHC-II⁺CD74⁺),
484 and long arrows indicate representative microglia expressing IFN response marker
485 (TMEM119⁺ISG15⁺). Results are representative of two independent experiments. Scale bar
486 = 100 microns.
- 487 c. Quantification of pro-inflammatory markers in brain tissue slices. Microglia were identified
488 based on TMEM119 expression and then scored for marker expression. Images (left panels)
489 show phenotype in representative tumor and distal regions. Tumor cells (ISG15⁺TMEM119⁻)
490 and other non-microglia cells (TMEM119⁻CD74⁻MHC-II⁻ISG15⁻) are shown in green and gray,

491 respectively. Pie graphs (right panels) show the proportion of microglia displaying marker
492 combinations. Frequencies are as follows: CD74⁺MHCII⁺ISG15⁺, distal < 0.01, tumor = 0.29;
493 CD74⁺MHC-II⁺ISG15⁻, distal < 0.01, tumor = 0.11; CD74⁻MHC-II⁺ISG15⁺, distal < 0.01, tumor
494 = 0.07; CD74⁺MHC-II⁻ISG15⁺, distal < 0.01, tumor = 0.07; CD74⁺MHC-II⁻ISG15⁻ distal = 0.01,
495 tumor = 0.08; CD74⁻MHC-II⁺ISG15⁻, distal < 0.01, tumor = 0.02; CD74⁻MHC-II⁻ISG15⁺, distal
496 = 0.01, tumor = 0.11; CD74⁻MHC-II⁻ISG15⁻, distal = 0.97, tumor = 0.24. n=4 mice per condition.

497 Scale bar = 100um.

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

502

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

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

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

510 c. Schematic of experimental design to compare disease progression in FIRE-WT and FIRE-
511 KO mice. FIRE-WT (n=19) and FIRE-KO (n=14) mice were injected intracranially with
512 100,000 GFP and luciferase (GFP-Luc) labeled EO771 cells. Control FIRE-WT mice (n=8)
513 were also injected with PBS. Animals were imaged for luminescence (IVIS) every three days
514 before dissection at endpoint on day 14.

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

- 517 e. Bar graph shows percentage body weight change for surviving PBS injected (n= 8), FIRE-
518 WT (n=19), and FIRE-KO (n=9) animal from **d** at day 14 relative to day 0. *P* values
519 determined by unpaired two-sided student's *t*-test and error bars represent mean +/- standard
520 deviation.
- 521 f. Bar graph summarizes the frequency of animals that displayed tumor growth and tumor
522 rejection in FIRE-WT and FIRE-KO mice. Tumor rejection was defined by a lack of
523 engraftment or engraftment followed by tumor rejection. *P* value was determined by two-
524 sided Fisher's exact test.

525

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

- 527 a. Schematic of experimental design to compare NK and T cell responses in FIRE-WT and
528 FIRE-KO EO771 tumor bearing mice. FIRE-WT (n=8) and FIRE-KO (n=8) mice were injected
529 intracranially with 100,000 EO771 GFP-Luc cells. Animals were imaged for luminescence
530 (IVIS) on day 1, 4 and 6 before dissection on day 7.
- 531 b. Analysis of NK and T cell subsets in FIRE-WT (n=7) and FIRE-KO (n=8) mice by flow
532 cytometry. Representative FACS plots show gating for each NK and T cell subset after gating
533 for single, live (Sytox negative) cells.
- 534 c. Bar graphs show cell counts for NK and T cell subsets. Counts shown are out of 100,000
535 single, live cells analyzed. *P* values are the result of a student's un-paired two sided *t*-test.
536 Error bars represent mean +/- standard deviation.
- 537 d. Analysis of CD107a expression in NK and CD8+ T cells by flow cytometry. FACS plots (left
538 panels) show expression of CD107a from spleen and brains of representative animals from
539 each cohort. Bar graph shows cell counts out of 100,000 single, live cells. *P* values are the
540 result of a student's un-paired two sided *t*-test. Error bars represent mean +/- standard
541 deviation.

542

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

- 544 a. Schematic of experimental design to determine effects of T cell deficiency on BCBM. Tumor
545 burden was compared in three cohorts of animals, FIRE-WT vehicle treated (Veh., n=13),
546 FIRE-WT FTY720 treated (FTY, n=6), and RAG1-KO (RAG1, n=12). Vehicle (PBS + 0.1%
547 DMSO) or FTY720 (5mg/kg) were administered via intraperitoneal injection to FIRE-WT
548 animals on day 0 and repeated daily. 70,000 EO771 GFP-Luc cells were delivered to each
549 animal in all three cohorts by intracranial injection on day 0 following drug delivery. Brain
550 tissues were harvested at endpoint on day 12 and analyzed for tumor burden by IVIS and
551 immune response by flow cytometry.
- 552 b. Bar graph shows the percentage of TCRb⁺ T cells in brain tissues harvested from each cohort
553 (n=6 Veh., n=6 FTY, n=4 RAG1) of animals at endpoint, gated out of single, live (sytox
554 negative) CD45^{hi} cells as shown in **Extended Data Figure 6a,b**. *P* values shown are the result
555 of an unpaired two sided student's *t*-test. Error bars represent mean +/- standard deviation.
- 556 c. Quantification of EO771 tumor engraftment at endpoint on day 12 by IVIS. Bar graph shows
557 frequency of animals in vehicle (Veh.), FTY720 (FTY), and RAG1-KO (RAG1) groups that
558 grew tumors. *P* = 0.51 Veh. vs FTY. and *P* = 0.21 Veh vs RAG1 by two-sided Fisher's exact
559 test.
- 560 d. Quantification of EO771 tumor burden at endpoint on day 12 by IVIS. Box and whisker plots
561 show total flux per brain of vehicle (Veh.) and FTY720 (FTY) injected FIRE-WT and RAG1-
562 KO (RAG1) cohorts. Bounds of box and whiskers are indicative of the first through fourth
563 interquartile range. *P* values shown are the result of an unpaired two sided student's *t*-test.
- 564 e. Analysis of pro-inflammatory marker expression in microglia from T cell deficient mice (n=6
565 Veh., n=6 FTY, n=4 RAG1). FACS plots (left panels) show expression of MHC-II, CD74 and
566 BST2 in representative animals, following gating on single, live (sytox negative)
567 CD45^{int}CD11b⁺Ly6c^{neg} microglia as shown in **Extended Data Figure 6b**. Bar graphs (right
568 panels) show the percentage of marker positive microglia in each cohort. *P* values are the

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

571

572 **Figure 7: Altered microglia activation in animals lacking T cells**

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

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

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

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

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

587 f. Heat map of log₂ fold change of key markers of the secretory (Secr, top), IFN response (IFNR,
588 middle), and antigen presentation (AP, bottom) programs separated by timepoint and mouse
589 strain.

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

594

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

- 597 a. Schematic shows experimental design for scRNA-seq of human microglia from humanized
598 MITRG mice transplanted with 231BR cells. MITRG mouse pups were injected with GFP-
599 labeled iPSCs, aged to 10 weeks and injected intracardiac with mCherry-labeled 231BR
600 cells. Brains from control (n=3) and metastatic (n=3) mice were digested to single cell
601 suspensions three weeks later. Dissociated cells from each sample were indexed using
602 MULTI-seq. Mouse cells were removed using anti-mouse MHC-I magnetic beads, and
603 recovered cells were pooled into metastatic or control samples for scRNA-seq.
- 604 b. tSNE plot shows human cells (n=21,353) colored by mouse and labeled by cell type. Top
605 marker genes (gray) for each cell type were identified using the Wilcoxon rank sum test. See
606 **Supplementary Table 6** for full marker gene list. pvMacro=perivascular macrophages.
- 607 c. Bar plot shows selected top GO terms associated with the human BCBM microglia response
608 signature. DE genes (n=4,904, adjusted $p < 0.05$) were determined using the Wilcoxon rank
609 sum test. GO terms were determined using Enrichr and select upregulated terms with P values
610 < 0.05 were retained. See **Supplementary Table 6** for full gene list.
- 611 d. Kaplan-Meier plot shows overall survival probability in human BCBM patients stratified by
612 expression of canonical microglia genes. Bulk RNA-seq data from patient BCBM tumors
613 (n=20, Varešlija et al, 2018)⁵⁵ was scored for microglia gene signature and stratified into high
614 and low groups. Scores were determined using the sum of scaled and centered values from
615 $\log(\text{CPM} + 1)$ transformed data.
- 616 e. Model for role of microglia in promoting anti-tumor immunity. In microglia replete conditions (+
617 Microglia), microglia respond to BCBM by upregulating proinflammatory programs (IFN
618 response, antigen presentation (AP), and secretory) that promote anti-tumor CD4, CD8, and
619 NK cell responses and tumor regression in the CNS. In microglia depleted conditions (-
620 Microglia), NK and T cell responses are deficient and the proportion of Tregs is increased,

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

625

626

627

628 **References**

629

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775

776 **Methods**

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

781

782 **Normal human brain and human BCBM samples**

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

787

788 **Cell lines**

789 MDA-MB-231-Br2²⁶ cells stably transduced with membrane targeted AcGFP
790 (rLV.EF1.AcGFP1Mem-9, ClonTech/Takara Bio, USA, Cat#0019VCT), mCherry
791 (rLV.EF1.mCherry-9,
792 ClonTech/Takara, Cat#0037VCT), and luciferase lentivirus were a gift from Ian Smith
793 (Parker, 2017, Bos, 2009). 4T1 cells were purchased from ATCC (ATCC Cat# CRL-2539,
794 RRID:CVCL_0125), stably infected with GFP lentivirus (Santa Cruz Biotechnology, copGFP
795 Control Lentiviral Particles, Cat#sc-108084) at a MOI of 10, and sorted for GFP expression. EO771
796 cells were purchased from CH3 Biosystems (Cat. No. 94A001, RRID:CVCL_GR23) and stably
797 infected with pCDH-EF1a-eFFly-eGFP) lentivirus particles. pCDH-EF1a-eFFly-eGFP was a gift

798 from Irmela Jeremias (Addgene plasmid #104834; <http://n2t.net/addgene:104834>;
799 RRID:Addgene_104834). To produce lentiviral particles, HEK293T cells were transfected with
800 pCDH-EF1a-eFFly-eGFP together with pMD2G and psPAX2 packaging plasmids using
801 Lipofectamine 2000 (Invitrogen, Cat# 11668027). Supernatants containing lentiviral particles were
802 used to infect EO771 cells overnight in the presence of 8 µg/ml polybrene (Sigma-Aldrich, Cat#
803 TR-1003-G). Transduced EO771 cells were sorted by GFP expression on a BD FACSAria Fusion
804 cell sorter. MDA213-BRm and 4T1 cell lines were cultured in DMEM, 5% FBS, 10U/ml penicillin,
805 0.1mg/mL streptomycin (GE Healthcare Cat#SV30010), at 37 °C, 5% CO₂, 95% relative humidity.
806 EO771 cells were cultured in RPMI 1640, 5% FBS, 10U/ml penicillin, 0.1mg/mL streptomycin,
807 10mmol/L HEPES at 37 °C, 5% CO₂, 95% relative humidity. All cell lines were authenticated by
808 STR analysis by ATCC prior to injection.

809

810 **Mouse strains**

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

823

824 **Immunofluorescence analysis of human BCBM samples**

825 4- μ m sections were heated at 65 °C and deparaffinized in Histo-Clear (National Diagnostics, #HS-
826 200, Atlanta, Georgia, USA). Tissues were rehydrated with graded solutions of ethanol (100%-
827 50%). Antigen retrieval was performed using a microwave pressure cooker with 10 mM citric acid
828 buffer (0.05% Tween-20, ThermoFisher Scientific Cat#BP337500, pH 6.0). Tissues were blocked
829 in blocking solution (0.1% Tween-20 and 10% Goat Serum in PBS), incubated with primary
830 antibodies diluted in blocking solution at 4 °C overnight, washed in PBS, incubated with secondary
831 antibodies diluted in blocking solution for
832 one hour at room temperature. Slides were mounted with VECTASHIELD Antifade Mounting
833 Medium with DAPI (Vector Laboratories, #H-1200, Burlingame, California, USA) and micrographs
834 were taken with the BZ-X700 Keyence fluorescence microscope.

835

836 **Generation of BCBM in mice**

837 Intracardiac injections were performed as described by Campbell et al, 2012⁶³ into the left cardiac
838 ventricle of anesthetized mice (300mg/kg Avertin). For 231BR brain metastasis 500,000 cells in
839 100 μ L of DPBS were injected into nine-week-old Foxn1^{nu/nu} or 10-week-old MITRG mice. For 4T1
840 brain metastasis, 100,000 cells were injected into nine-week-old BALB/cJ mice in 100 μ L of DPBS.
841 For the intracranial injection of FVB, C57BL/6, FIRE-WT, FIRE-KO, RAG1-KO or albino B6,
842 100,000 VO-PyMT, EO771, or Py8119 cells were injected in 10 μ L PBS to a depth of 3mm into
843 the right coronal suture of five-week-old mice^{18,20}. Tumors were not allowed to exceed 1.7mm
844 along any diameter or 10% of the animal's body weight in accordance with IACUC protocol.
845 Control mice were injected with 10 μ L PBS. Injections were replicated in 2-3 cohorts of 4-6 mice
846 and in different mouse strains to ensure reproducibility of results.

847

848 **Dissection and visualization of mouse BCBM by whole mount fluorescence microscopy**

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

853

854 **Mouse brain fixation and sectioning**

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

859

860 **Immunofluorescence staining of floating sections**

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

871 Cat#A11034); Goat anti-rat IgG conjugated with Alexa Fluor 568 and 647 (RRID: AB_2534074

872 Cat#A11006 and RRID: AB_141778 Cat#A21247); Goat anti-hamster conjugated with Alexa Fluor
873 647 (RRID: AB_2535868 Cat#A21451) (Thermo Fisher Scientific Inc., Carlsbad, California, USA).
874

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

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

883

884 **CODEX Imaging of Mouse BCM**

885 C57BL/6 control or EO771-GFP tumor bearing mouse brain tissue was prepared and
886 immunostained following CODEX manual rev. C, and imaged using 20X PlanApo 0.75 NA lens
887 on the CODEX automated imaging system with the Keyence 700 microscope and BZX software.
888 A 7X7 tile scan with 6x Z planes 1.5u steps was taken and processed using the CODEX processor
889 1.8. The output images were segmented in QuPath using StarDist
890 (<https://github.com/stardist/stardist>). Segmented cells were phenotyped by a user trained
891 machine learning classifier in QuPath based on the marker expression for TMEM119, IBA1, MHC-
892 II, CD74, ISG15. The following antibodies, barcode/reporter and fluorescence combinations were
893 used:

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

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

901

902 **Isolation of cells for scRNA-seq**

903 Single cell suspensions from mouse brains were prepared using the Adult Brain Dissociation Kit,
904 Mouse and Rat (Miltenyi Biotec) with some modifications. Whole dissected brains were partitioned
905 and placed into C tube (Miltenyi Biotec, Cat#130-093-237) containing enzyme P and A (Foxn1^{nu/nu}
906 mice) or 1mg/mL Collagenase D (Millipore Sigma Cat#11213857001, C57BL/6 and RAG1-KO
907 mice. Brain tissue was digested using gentleMACS Octo Dissociator with heaters operating the
908 37°C adult brain dissociation protocol. After removal of myelin by density centrifugation, remaining
909 red blood cells were lysed. Cells were blocked with anti-CD16/32 and stained with fluorescent
910 antibodies.. C57BL/6 and RAG1-KO cells from individual mice were labeled using a CellPlex
911 multiplexed oligo labeling kit in accordance with the manufacturer's protocol (10x Genomics Cat#
912 1000261). The labeled cells were sorted on a BD FACSAria Fusion sorter. For sorting of microglia,
913 astrocytes, cancer cells, and total leukocytes, cells were gated for size based on forward and side
914 scatter, single cells, and Sytox Blue viability (Thermofisher, Cat#S34857. All myeloid cells (CD45⁺
915 CD11b⁺) and astrocytes (CD45⁻, ACSA2⁺) were sorted from control and metastatic mouse brains,
916 GFP⁺ 231BR cells were sorted from metastatic brains, and total leukocytes (CD45⁺) were sorted
917 from tumor-bearing control and RAG1-KO mouse brains into 500µL of chilled FACS buffer.

918

919 **scRNA-seq of murine brain leukocytes**

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

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

929

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

931 Tissue was prepared as for FACS sorting using 1mg/mL Collagenase D (Milipore Sigma
932 Cat#11213857001) for digestion. Cells were stained with ZombieNIR viability dye (1:500,
933 BioLegend Cat. No. 423106), and blocked with anti-CD16/32 antibody. Next, cells were stained
934 with fluorescent antibodies for extracellular markers and analyzed using BD Fortessa X20 and
935 FlowJo v10 software. For intracellular staining of Foxp3 and CD3e, cells were fixed with the
936 eBioscience Foxp3 /Transcription Factor Staining Buffer set according to the manufacturer's
937 instructions (ThermoFisher Cat #00-5523-00) for analysis on the BD Fortessa X20. The following
938 antibodies were used for flow cytometry analysis: CD45-BV510 (Biolegend, 30-F11,
939 1:100), CD45-FITC (Biolegend, 30-F11, 1:100), CD11b-BV605 (Biolegend, M1/70, 1:200),
940 CD11b-PE (Biolegend, M1/70, 1:200), CD11b-BV650 (Biolegend, M1/70, 1:200), ACSA-2-APC
941 (Miltenyi, REA969, 1:80), Ly6C-BV785 (Biolegend, HK1.41:200, 1:200), Ia/Ie (MHC-II)PacificBlue
942 (Biolegend, M5/114.15.2, 1:500), CD74-AF647 (Biolegend, In1/CD74, 1:100),
943 CD317-PE (Biolegend, 129C1, 1:100), CD3-PerCPCy5.5 (Biolegend, 17A2, 1:100), TCRb-
944 PECy5 (Biolegend, H57-597, 1:100), NK1.1-PEDazzle594 (Biolegend, PK136, 1:100),
945 CD4BV605 (Biolegend, RM4-5, 1:500), CD8a-PacificBlue (Biolegend, 53-6.7, 1:500), Foxp3-PE
946 (eBioscience, FJK-16s, 1:100), CD152 (CTLA-4)-APC (BD, UC10-4F10-11, 1:100), CD44-PECy7

947 (Biolegend, IM7, 1:100), CD62L-BV785 (Biolegend, MEL-14, 1:100), CD107a-FITC (Biolegend,
948 1D4B, 1:100).

949

950 ***In vitro* differentiation and early postnatal transplantation of iHPCs**

951 Differentiation of Hematopoietic Progenitor Cells from iPSCs (iHPCs) performed according to
952 McQuade et al. (2018). Briefly, iPSCs were first passaged in mTeSR-E8 and transferred to
953 Medium A from the STEMdiff Hematopoietic Kit (Stem Cell Technologies, Cat#05310). On day
954 three, flattened endothelial cell colonies were transferred to Medium B for seven days. On day
955 10, non-adherent CD43⁺ iHPCs were collected and frozen in Bambanker (Fisher Scientific,
956 Cat#NC9582225) for later transplantation. Cells were thawed in iPS-Microglia medium
957 (DMEM/F12, 2X insulin-transferrin-selenite, 2X B27, 0.5X N2, 1X glutamax, 1X non-essential
958 amino acids, 400 mM monothioglycerol, and 5 mg/mL human insulin freshly supplemented with
959 100ng/mL IL-34,
960 50ng/mL TGFb1, and 25 ng/mL M-CSF (Peprotech, Cat#100-21) according to McQuade et al,
961 2018⁵². Early Postnatal Intracerebroventricular Transplantation of iHPCs was performed as
962 described in Hasselmann et al, 2019⁵³.

963

964 **Isolation of human xenotransplanted microglia**

965 10-week-old, MITRG mice were injected intracardially with 500,000 mCherry labeled 231BR cells
966 as previously described. 25 days post-injection, following perfusion with ice cold PBS containing
967 5µg/ml actinomycin D (act D, Cat#A1410), whole metastatic brains were imaged on a dissection
968 microscope (Leica Biosystems, DMC 2900) for mCherry and GFP intensity. Half brains were
969 dissected, fixing the left hemisphere in 4% PFA for histology. The right hemisphere was prepped
970 for dissociation as described in Hasselmann et al, 2019⁵³ with modifications. The cerebellum was
971 removed and the whole right hemisphere was stored briefly in RPMI 1640 containing 5µg/mL act

972 D, 10 μ M triptolide (Sigma-Aldrich, Cat#T3652), and 27.1 μ g/mL anisomycin (Sigma-Aldrich,
973 Cat#A9789). Tissue dissociation was performed using the Tumor Dissociation kit, human (Miltenyi
974 Biotec) as previously described with the kit's enzymes, 5 μ g/mL act D, 10 μ M triptolide, and
975 27.1 μ g/mL anisomycin using the preprogrammed soft tumor protocol.. Myelin and debris removal
976 was performed in 8mL 23% Percoll (GE Healthcare, Cat#45-001-748), overlaid with 2mL of 1X
977 DPBS.

978

979 **MULTI-seq labeling and scRNA-seq of human microglia**

980 Individual mice were barcoded following the "MULTI-seq lipid- tagged indices for sample
981 multiplexing for scRNAseq" protocol⁶⁴. Lipid anchor and co-anchor reagents were a gift from Zev
982 Gartner, and barcode index oligos were purchased from Integrated DNA Technologies, Inc. Mouse
983 cell removal was performed using mouse cell removal beads (Miltenyi Biotec) separated using LS
984 columns and the MidiMACs separator (Miltenyi Biotec). Control and metastatic samples were then
985 pooled separately. Cells were resuspended to ~1,000 cells per microliter in FACS buffer,
986 according to counts performed on a hemocytometer.

987

988 **ScRNA-seq of MITRG human microglia**

989 Final cell suspensions were counted on the Countess II automated cell counter to determine
990 actual concentration for droplet generation. Cells were loaded onto the 10x Genomics Chromium
991 Single Cell Gene Expression 3' v3 Chemistry kits for GEMs generation. Following the Chromium
992 Single Cell 3' Reagents Kits version 3 user guide (CG000183 Rev C), cells were loaded to achieve
993 10,000 cells for capture. MULTI-seq barcode libraries were prepared according to the MULTI-seq
994 protocol⁶². Libraries were sequenced on the Illumina NovaSeq 6000 platform to achieve an
995 average read depth of 50,000 mean reads per cell for 3' gene expression libraries.

996 MULTI-seq barcode libraries were sequenced to achieve at least 5,000 reads per cell.

997 Sequencing reads were aligned utilizing 10x Genomics Cell Ranger Count 3.1.0 to a dual indexed
998 GRCh38 and mm10 reference genome. All libraries were aggregated using 10x Genomics Cell
999 Ranger Aggr 3.1.0, to normalize the number of mean reads per cells. MULTI-seq reads were
1000 processed according to the MULTI-seq protocol ([https://github.com/chris-mcginnis-](https://github.com/chris-mcginnis-ucsf/MULTIseq)
1001 [ucsf/MULTIseq](https://github.com/chris-mcginnis-ucsf/MULTIseq)).

1002

1003 **Analysis of BCBM in FIRE mice**

1004 Four six-week-old *Csf1r*^{ΔFIRE/ΔFIRE} (FIRE-KO) and *Csf1r*^{FIRE/FIRE} (FIRE-WT) mice were injected
1005 intracranially in the right coronal suture with 100,000 enhanced GFP and luciferase labeled
1006 EO771 cells as previously described. Mice were imaged for luciferase luminescence one day after
1007 injection, and every three days thereafter until endpoint. Imaged mice were anesthetized via
1008 isoflurane inhalant and administered 300μg D-Luciferin (Goldbio), intraperitoneally, in sterile
1009 DPBS. Following a 10-minute incubation, mice were imaged for bioluminescence utilizing an IVIS
1010 Lumina III In Vivo Imaging System (Xenogen). Regions of interest were selected around each
1011 brain and average photon flux (total photons/s-
1012 cm²) was recorded using Living Image analysis software (RRID:SCR_014247,
1013 <http://www.perkinelmer.com/catalog/category/id/living%20image%20software>) with average
1014 background flux subtracted. At endpoint, mice were weighed, euthanized, and dissected and
1015 whole brains were removed and placed in a 24 well tissue culture plate submerged in ice cold
1016 PBS with D-Luciferin (1.5 mg/mL, Goldbio, Cat# LUCK-1G). After 10 minutes incubation, whole
1017 brains were placed on a black plastic card and imaged for luminescence for 1 second. A region of
1018 interest was drawn around each brain and the total flux (ptotal photons/s-cm²) was recorded for
1019 analysis.

1020

1021

1022 **Fingolimod (FTY720) HCL dosing**

1023 FTY720 was purchased from Selleckchem (Cat. no. S5002), and reconstituted to a final
1024 concentration of 1mg/mL FTY720 (0.001% DMSO). Mice were injected intraperitoneally with 50-
1025 100uL of vehicle or 5mg/kg FTY720 immediately before intracranial injection with 70,000 EO771
1026 eGFP eFFly cells, and daily for the duration of the experiment. The experimental groups were
1027 blinded when performing intracranial injections of EO771 cells.

1028

1029 **Cytokine screen of Microglia**

1030 Control and 14-day EO771 eGFP eFfly tumor bearing brains were digested into single cell
1031 suspension as previously described for flow cytometry. Cells were immunostained for BV510-
1032 CD45 (Biolegend clone 30-F11, 1:100), BV605-CD11b (Biolegend clone M1/70, 1:200), BV785-
1033 Ly6C (Biolegend clone HK1.4, 1:200), and PE/Dazzle594-NK1.1 (Biolegend clone PK136, 1:100).
1034 50,000 microglia per sample were FACS sorted based on CD45^{lo}, CD11b⁺ Ly6C⁻ NK1.1-
1035 expression into FACS buffer. Cells were resuspended in 100uL of 150mM sodium chloride, 1%NP-
1036 40 50mM Tris pH 8.0 cell lysis buffer containing 1X Halt Protease Inhibitor (Thermo Fisher Cat.
1037 #78430) and stored frozen at -80°C. Frozen samples were shipped on dry ice to Eve Technologies
1038 Corp (3415A - 3 Ave.,
1039 NW, Calgary, AB T2N 0M4) to perform a standardized mouse cytokine array / chemokine array
1040 31-Plex (MD31, Millipore MILLIPLEX). The analytes tested for include Eotaxin, G-CSF, GM-CSF,
1041 IFN γ , IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70),
1042 IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1 α , MIP-1 β , MIP-2,
1043 RANTES, TNF α , VEGF.

1044

1045 **Human/mouse cell assignment**

1046 Cells were aligned to a merged GRCh38/mm10 genome using Cell Ranger v3. Cell species was
1047 determined based on the frequency of reads aligning to the mouse genome with very low-quality
1048 cells with <200 genes (nFeature_RNA) filtered before estimating. Cells were called as mouse for
1049 all cells above the top elbow in the mouse read mapping frequency plot (>0.875 for Foxn1^{nu/nu}
1050 data; >0.95 for MITRG data), human for all cells below the bottom elbow (<0.05 for Foxn1^{nu/nu}
1051 data; <0.1 for MITRG data), and any other cells were discarded as doublets or poor quality. Any
1052 counts for GRCh38 genes in the cells called as mouse were removed from the expression matrix
1053 and vice versa for mm10 genes in human cells.

1054

1055 **Quality control metrics**

1056 Cells for the Foxn1^{nu/nu} cell type identification analysis were filtered to have between 500 and 2000
1057 genes (nFeature_RNA) and <10% mitochondrial genome reads (percent.mito) in any retained
1058 cell. Cutoffs were selected based on analysis of violin plots for visual outliers. Putative
1059 microglia/astrocyte doublet clusters with marker gene co-expression were removed from the
1060 Foxn1^{nu/nu} microenvironment. This cell set was then used for subset myeloid and astrocyte
1061 analyses based on the cell type labels. Cells were filtered for the myeloid analysis to have <5%
1062 percent.mito and low ribosomal expression (<10% of their transcriptome representing Rps and
1063 Rpl genes). Cells for the MITRG analysis were filtered to have <20% percent.mito. Doublets and
1064 empty gems (Negative) were removed from the MITRG analysis based on MULTI-Seq barcoding
1065 label assignment from the R package deMULTIplex. Cell cycle signatures (S.Score and
1066 G2M.Score, determined by CellCycleScoring in Seurat) were regressed from the data for the
1067 231BR analysis as well as the MITRG analysis before clustering and dimensionality reduction.

1068

1069 **Clustering and differential expression**

1070 Main clustering and dimensionality reductions were performed in Seurat using the default Louvain
1071 and tSNE methods. UMAP was used for dimensionality reductions in microglia subclustering
1072 analyses to better visualize global relationships. Some datasets were integrated using the mutual
1073 kNN algorithm adaptation in Seurat before these steps. Specifically, integration was performed on
1074 the Foxn1^{nu/nu} full microenvironment and astrocyte analyses by sequencing batch (Con1:Met1,
1075 Con2:Con3, Met2:Met3). Integrated analyses used the “vst” selection method with
1076 nfeatures=2000 for FindVariableFeatures and dims=1:30 for FindIntegrationAnchors and
1077 IntegrateData. Differential expression analyses were run on the RNA assay in Seurat with
1078 FindAllMarkers/FindMarkers using the Wilcoxon rank sum test and adjusted *P* values represent
1079 the Bonferroni corrected values for all single-cell analyses. Cell types and states were assigned
1080 to clusters manually based on gene expression profiles. All plotting functions through Seurat utilize
1081 ggplot2.

1082

1083 **GO term analysis and gene scoring**

1084 GO term analyses were performed using the MouseMine³² web portal with list input for M.
1085 musculus with the default background population for mouse analyses and using the Enrichr
1086 portal^{65,66} with a gene list input. Gene inputs included only genes considered differentially
1087 expressed with a Bonferroni adjusted *P* value < 0.05 from the Wilcoxon rank sum test. GO terms
1088 were selected from the Gene Ontology Enrichment section for biological_process with Holm-
1089 Bonferroni adjusted *P* value < 0.05 in MouseMine or the GO Biological Process 2018 list in Enrichr
1090 with unadjusted *P* value < 0.05. All gene scoring on singlecell data was performed in Seurat using
1091 the AddModuleScore function with default parameters.

1092 MG score gene list was taken directly as the Core MG list from Supplementary Table 3 in Bowman
1093 et al, 2016³⁰.

1094 Topic scores were determined for the MITRG mouse using the top 25 marker genes of each topic
1095 (ExtractTopFeatures with method = "poisson", options="min", and shared = FALSE), translated
1096 to human using the biomaRt package.

1097

1098 **Latent Dirichlet Allocation, Topic model**

1099 To fit a topic model using Latent Dirichlet Allocation (LDA), we used the R package 'CountClust'³³
1100 which was optimized for use on RNA-seq datasets. As input to our model, we provided a raw
1101 counts matrix containing all cells labeled as microglia and all detected genes from our Foxn1^{nu/nu}
1102 dataset. The topic model was fit using the 'FitGoM' function, with a range of cluster numbers (K),
1103 and an error tol = 10. We chose the model with K = 15 since it achieved a relatively low value for
1104 the Bayesian Information Criterion (BIC) and had enough resolution to provide topics with unique,
1105 biologically interpretable gene lists. Top gene markers for each topic were identified using the
1106 function 'ExtractTopFeatures' with method='poisson', options='min' and shared=TRUE for the
1107 marker heatmap (or shared = FALSE for gene scores).

1108

1109 **Pseudotemporal Ordering of Cells**

1110 Monocle 3⁶⁷ was used for trajectory inference and pseudotemporal ordering of cells. For input,
1111 the final annotated Seurat object was converted to a Monocle 3 cds object using the
1112 SeuratWrappers function as.cell_data_set. The counts data matrix was then processed with the
1113 standard Monocle 3 pipeline using default parameters in the preprocess_cds and
1114 reduce_dimension functions. Clustering was performed with cluster_cells using resolution=3e-4
1115 to maintain similar cluster assignments between Seurat and Monocle 3. The principal graph was
1116 constructed with the learn_graph function using one partition. To identify the root principal point
1117 for ordering cells, the helper function get_earliest_principal_node was used as defined in the
1118 Monocle 3 vignette, using D4_BL6 cells in our timepoint_strain metadata column.

1119 Pseudotemporal ordering was then performed using `order_cells` with
1120 `root_pr_nodes=get_earliest_principal_node(cds)`. To display the pseudotime data on the original
1121 Seurat UMAP embeddings, the Monocle `cds` object was converted to a Seurat object with the
1122 Seurat function `as.Seurat` and the pseudotime metadata column of the resulting object was
1123 transferred to the original Seurat object using `AddMetaData`. The pseudotime results were
1124 displayed as a feature plot using the plasma color palette from the `viridis` library.

1125

1126 **Survival analysis**

1127 Survival analysis was performed using the Brain-Met samples from Vareslija et al, 2018⁵⁵,
1128 based on the column header "M_" from their Github uploaded counts matrix
1129 (https://github.com/npriedig/jnci_2018/blob/master/brainMetPairs.salmon.cts.txt). This subset of
1130 the counts matrix was converted to $\log(\text{cpm} + 1)$ using the 'cpm' function in `edgeR`. This matrix
1131 was then merged with the clinical information from Table 1 of Vareslija et al, 2018⁵⁵, resulting in
1132 20 total samples (samples "7M_RCS" and "19.2M_Pitt" were dropped, the first due to a lack of
1133 matching clinical data and the second due to sample replication). Our genes and signatures of
1134 interest, CD74, BST2, MHC-II genes ("HLA-DMA", "HLA-DMB", "HLA-DOA", "HLA-DOB",
1135 "HLADPA1", "HLA-DPB1", "HLA-DQA1", "HLA-DQA2", "HLA-DQB1", "HLA-DQB2", "HLA-DRA",
1136 "HLA-DRB1", "HLA-DRB3", "HLA-DRB4", "HLA-DRB5") and microglia ("P2RY12", "TMEM119",
1137 "GPR34", "CX3CR1", "CD81", "SELPLG") were converted to their Ensembl IDs using 'mapIds'
1138 with `multiVals = 'list'` from `org.Hs.eg.db`, and added to the dataset as $\log(\text{cpm} + 1)$ for single genes,
1139 and the sum of the scaled data (z-scores) for multigene signatures. Survival analysis was
1140 performed using the R package `survminer`, where data stratification was made using
1141 'surv_cutpoint' and 'surv_categorize' to identify an optimal split, and the KM curves generated
1142 using 'survfit' in 'ggsurvplot'.

1143

1144 **Statistics and Reproducibility**

1145 No statistical methods were used to pre-determine sample sizes but sample sizes are similar to
1146 those reported in previous publications. Data distribution was assumed to be normal but this was
1147 not formally tested. Mice with insufficient viable cell yield were excluded from analysis by flow
1148 cytometry. Where possible, experimental groups were randomized prior to initiation of
1149 experiments. Investigators were not blinded to allocation and experimental outcome except where
1150 otherwise indicated.

1151

1152 **Data Availability**

1153 RNA-seq data that support the findings of this study have been deposited in the Gene
1154 Expression Omnibus (GEO) under accession codes GSE147949 and GSE237386. Reference
1155 genome GRCh38/mm10 are available from Ensembl. MULTI-seq reads were processed
1156 according to the MULTI-seq protocol⁶² and available on GitHub ([https://github.com/chris-](https://github.com/chris-mcginis-ucsf/MULTIseq)
1157 [mcginis-ucsf/MULTIseq](https://github.com/chris-mcginis-ucsf/MULTIseq)). Qptiff images were segmented in QuPath using StarDist and is
1158 available on GitHub (<https://github.com/stardist/stardist>).” All other data are available from the
1159 corresponding author on reasonable request.

1160

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